REMARKS

In the Final Action dated February 15, 2005, claims 33, 34 and 36-38 are pending and under examination. Claims 37-38 are objected to for certain alleged informalities. Claim 36 is rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite. Claims 36-38 are rejected under 35 U.S.C. §102(b) as allegedly anticipated by Thomson et al. (*Science* 282: 1145-1147, 1998). Claims 36-38 are also rejected under 35 U.S.C. §102(e) as allegedly anticipated by Thomson (U.S. Patent No. 6,200,806). Claims 33 and 34 are rejected under 35 U.S.C. §102(b) as allegedly anticipated by Vajta et al. (*Acta Vet Scan* 1997 or *Mol Reprod Dev* 1998).

This Response addresses each of the Examiner's objections and rejections. Applicants therefore respectfully submit that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Claims 37-38 are objected to for reciting the abbreviated term "hES".

Applicants have amended claim 37 such that the first appearance of "hES" is in parenthesis and follows the full name of the term. Withdrawal of the objection to claims 37-38 is therefore respectfully requested.

Claim 36 is rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite. Specifically, the Examiner contends that the reference to Example 6 renders the claim indefinite.

In response, claim 36 has been amended to delete the reference to Example 6 and to depend from claims 33-34. Support for this amendment is found in the specification, e.g., on page 30 and page 37. It is respectfully submitted that claim 36, as presently amended, is not indefinite. Withdrawal of the rejection of claim 36 under 35 U.S.C. §112, second paragraph, is therefore respectfully requested.

Claims 36-38 are rejected under 35 U.S.C. §102(b) as allegedly anticipated by Thomson et al. (*Science* 282: 1145-1147, 1998) (hereinafter "Thomson et al. (Science)").

The Examiner has interpreted claims 36-38 to encompass a human embryonic stem (hES) cell wherein the cell is cryopreserved. The Examiner contends that Thomson et al. (Science) teach that the cell lines disclosed in the reference were cryopreserved, and that after freezing the cell lines could be propagated without any apparent effect on the pluripotent characteristics of the cells. Thus, the Examiner contends that the claimed products and the cells disclosed by Thomson et al. (Science) are identical or substantially identical, unless Applicants prove otherwise.

Applicants respectfully submit that the claims, as presently amended, are directed to hES cells and hES cell compositions that are vitrified and have undergone the process of vitrification. Support for the amendment is found in the specification, e.g., on page 30, lines 20-23. Applicants respectfully submit that Thomson et al. (Science) do not teach or suggest vitrified hES cells or hES cell compositions.

In this connection, Applicants respectfully submit that the process of vitrification is a form of cryopreservation. The general term "cryopreservation" means to preserve cells in whole tissue by cooling to low subzero temperatures. However, the cells being preserved by some methods of cryopreservation are often damaged during the approach to low temperatures or warming to room temperature.

Various methods have been used to achieve cryopreservation. One method is vitrification and another method is controlled rate freezing. Each method has certain advantages and neither has been used to cryopreserve embryonic stem cells prior to the present invention.

Vitrification is a process of freezing which involves rapid "flash freezing" without the formation of damaging ice crystals. The process involves the use of cryoprotectants, which cause water to harden like glass, minimizing damage to materials/cells being frozen. Under such rapid cooling, water molecules do not have time to arrange themselves into a crystalline lattice structure. Equally important, the vitrification process completely avoids ice crystal formation in cryopreserved cells during warming/thawing to recover the material/cells for biological applications. Importantly, other cryopreservation methods in general give rise to crystallisation and hence material/cell damage during cooling and warming. However, vitrification avoids this problem. Applicants are providing herewith a copy of Kuleshova and Lopata, *Fertility and Sterility* 78(3):449-454 (2002) (**Exhibit A**), which includes the definition and history of vitrification.

The controlled rate freezing method takes the sample gradually through to freezing generally at 1 to 2°C per minute. The rate of cooling leads to extracellular crystal formation and also toxic damage. Once cell junctions are destroyed, the cells detach from the clumps and the clumps cannot be recovered after thawing. In this connection, Applicants direct the Examiner's attention to Fujioka et al. (*Int. J. Dev. Biol.* 48: 1149-1154,2004) (enclosed as **Exhibit B**) on page 1149, second column, line 7-9, referencing cell death after thawing subsequent to controlled rate freezing.

Vitrification, on the other hand, equilibrates the sample with the cryotprotectants and then plunges the cell into liquid nitrogen and rapidly freezes the sample.

The main differences between vitrification and controlled rate freezing are the cooling rate and the cryoprotectants used. While the approach of vitrification can avoid the

formation of crystals, the high concentration of cryoprotectants may, under certain circumstances, induce significant toxic effects.

The two methods are distinctly different and yield different outcomes for cells, in particular for embryonic stem cells. Either could be applied. However, as described in the specification on page 30, lines 20-23, Applicants have found that vitrification is best in its application to embryonic stem cells.

Vitrification has not been successfully applied embryonic stem cells prior to the present invention. Subsequent to the filing of the present application, articles such as Fujoka *et al.* (**Exhibit B**) and Zhou *et al.* (*Chinese Medical J.* 117: 1050-1055, 2004, attached hereto as **Exhibit C**) have published, which relate to the cryopreservation of ES cells, specifically to the application of vitrification to ES cells. These articles have shown that vitrification is generally not considered desirable for preserving undifferentiated or differentiated ES cells due to toxic levels of cryoprotectant required in the cells. Zhou *et al.* also acknowledge that the first to apply vitrification to ES cells is Reubinoff et al., referencing *Human Reprod.* 16: 2187-2194 (2001). The three authors of Reubinoff et al. (2001), Reubinoff BE, Pera MF and Trounson AO, are co-inventors named in the present application.

As indicated by the Examiner, Thomson et al. (Science) disclose cryopreservation of ES cells. However, there is no disclosure in Thomson et al. as to the exact method of cryopreservation, nor is there any indication that vitrification was used. Therefore, Applicants respectfully submit that Thomson et al. do not teach or disclose vitrification for cryopreservation of hES cells. As discussed above, vitrification is a specific form or cryopreservation, which is distinct from other cryopreservation methods and yield products with superior properties.

Therefore, it is respectfully submitted that Thomson et al. (Science) do not teach the vitrified hES cells and compositions, as presently claimed. Withdrawal of the rejection of claims 36-38 under 35 U.S.C. §102(b) based on Thomson et al. (Science) is therefore respectfully requested.

Claims 36-38 are also rejected under 35 U.S.C. §102(e) as allegedly anticipated by Thomson (U.S. Patent No. 6,200,806). The Examiner's characterization of the '806 patent is essentially the same as that of Thomson et al. (Science).

Applicants respectfully submit that similar to Thomson et al. (Science), the '806 patent does not disclose vitrification for cryopreservation of hES cells, as presently claimed. Therefore, it is respectfully submitted that the '806 patent does not teach the vitrified hES cells and cell compositions, as presently claimed. Withdrawal of the rejection of claims 36-38 under 35 U.S.C. §102(e) based on the '806 patent is therefore respectfully requested.

Claims 33 and 34 are rejected under 35 U.S.C. §102(b) as allegedly anticipated by Vajta et al. (*Acta Vet Scan* 1997, or *Mol Reprod Dev* 1998).

Claim 33 is drawn to a method of preserving isolated differentiated or undifferentiated human embryonic stem cells by vitrification. Dependent claim 34 specifically indicates that the method is the Open Pulled Straw method of vitrification.

The Examiner contends that Vajta et al. teach an Open Pulled Straw method for the vitrification of human embryos. The Examiner considers that an embryo consists of cells differentiated from embryonic stem cells. Therefore, the Examiner concludes that Vajta et al. anticipate the claimed methods.

Applicants respectfully direct the Examiner's attention to the amendment to claim 33 to recite "isolated" human embryonic stem cells. Applicants respectfully submit that the

presently claimed isolated human embryonic stem cells, which are obtained through an artificial, *in vitro* culturing system, are distinct from an embryo and blastocyst, as well as distinct from cells within an embryo and blastocyst.

More specifically, Applicants submit that an hES cell in an *in vitro* environment is unlike any cell in any *in vivo* state and is not considered to be a natural phenomenon. In deriving the hES cells, the trophectoderm is removed to access the ICM, resulting in disruption of the "normal" embryonic development environment. That is, the ICM is now out of context with the trophectoderm and the blastocyst environment has been destroyed. When cells of the isolated ICM are plated, there is no longer a normal embryo. All media and subsequent supplements employed in culturing the cells are *in vitro* "approximates" that have been found to be supportive of hES and do not mirror a normal *in vivo* environment. Therefore, the hES cells so derived are artificial products.

Such artificial hES cells have properties not shared by cells within an intact embryo. For example, no pluripotent cells demonstrate long-term self-renewal *in vivo* and proliferate only briefly before becoming cells with a more restricted developmental potential. In contrast, the ES cells derived in culture are pluripotent and can indefinitely self renew.

All these facets of ES derivation call into question any assertion that these cells are the equivalent of cells of an early embryo. In a recent publication co-authored by J. Thomson (*Development* 132, 227-233, 2005, attached as **Exhibit D**), the following statement appears in the summary:

> Historically, ES cells have been believed to most closely resemble pluripotent primitive ectoderm cells derived directly from the ICM. However differences between ES cells and primitive ectoderm cells have caused developmental biologists to question whether ES cells really have an in vivo equivalent

or whether their properties merely reflect their tissue culture environment.

Therefore, Applicants respectfully submit that isolated embryonic stem cells are distinct from embryos and blastocysts, as well as from cells within embryos and blastocysts. Thus, the teaching of Vajta et al., which relate to cryopreservation embryos, do not anticipate the claimed methods directed to vitrification of isolated hES cells.

The Examiner has also made the comment that the instant specification indicates that the methodology contemplated is not new and makes specific reference to the methods disclosed by Vajta et al.

Applicants respectfully disagree with the Examiner's characterization of the instant disclosure. The specification does reference the cryopreservation methods disclosed by Vajta et al. as applied to embryos. However, the present invention is the first to apply the open pulled straw (OPS) vitrification method to isolated hES cells, derived *in vitro*. The disclosure of applying the open pulled straw (OPS) vitrification method to isolated hES cells is found only in the present application, not in Vajta et al.

Applicants further respectfully submit that the specification also clearly indicates that the open pulled straw (OPS) vitrification method was applied to hES cells with some modifications (see page 30, line 10 and 11). The OPS method in the Vatja *et al.* citation utilizes holding media (HM) comprising 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) dissolved in a medium (TCM-199 and 20% calf serum), or alternatively a medium comprising 18% EG, 18% DMSO and 0.6M sucrose. These conditions are clearly different from those employed in the vitrification method applied to human embryonic stem cells, as disclosed in the present application. As described on page 30 of the specification, the HM comprises (i) in VS1, 10% dimethylsulfoxide and 10% ethylene glycol; or alternatively (ii) in VS2, 20%DMSO,

20% EG and 0.5M sucrose. Both holding media include DMEM containing HEPES buffer supplemented with 20% foetal bovine serum. The conditions employed in the present application are clearly different from those disclosed by Vajta et al. and are specific for human embryonic stem cells derived in accordance with the present application. Claims 39-45 are added to define the holding medium employed in the instant vitrification process. Support for these claims is found in the specification, e.g., on page 30, lines 16-25. No new matter is introduced.

In view of the foregoing, it is respectfully submitted that the claimed methods, directed to vitrification of isolated human ES cells, are not taught by Vajta et al. Withdrawal of the rejection of claims 33-34 under 35 U.S.C. §102(b) based on Vajta et al. is therefore respectfully requested.

Applicants further respectfully submit that the present methods are not only novel, but also unobvious, in view of the disclosure of Vajta et al. In particular, as discussed above, human ES cells derived *in vitro* are artificial in nature and have distinct characteristics compared to cells of an embryo. There is no indication from Vajta et al. that human ES cells could be frozen and survive under the conditions disclosed therein in connection with embyros.

Applicants further respectfully submit that those skilled in the art would not have been motivated to employ the methodology of Vatja et al. to hES cells, and would not have had a reasonable expectation of success in applying the methodology of Vatja et al. to hES cells. In this connection, Applicants respectfully submit that cryopreservation of embryos, in general, was known at the time of the instant filing, to be associated with various detrimental effects on cell viability. For example, it was well recognized that cryopreservation of human embryos significantly decreased their viability, as illustrated by their reduced capacity for implantation

(Levran et al., 1990, attached hereto as **Exhibit E**). It was also shown that ultra rapid freezing (similar to vitrification) of mouse oocytes lowered the cell number in the inner cell mass of day 5 blastocysts (Van der Elst et al., 1998, attached hereto as **Exhibit F**). Further, it was reported that glucose uptake by early stage embryos (2-cell) was impaired by vitrification (Uechi et al., 1999, hereto as **Exhibit G**). Importantly, glucose uptake is predictive for blastocyst viability after thawing, according to Gardner et al., 1996, attached hereto as **Exhibit H**), suggesting that component cell viability was impaired. It was also postulated that vitrification caused a reduced blastocyst quality by affecting functional integrity (e.g., membranal glucose transporter damage) (Uechi et al., 1999, attached hereto as **Exhibit G**). In addition, reagent components of vitrification solutions essential to the process of vitrification were known to be potentially harmful to embryo development (Kasai et al., 1990, attached hereto as **Exhibit I**).

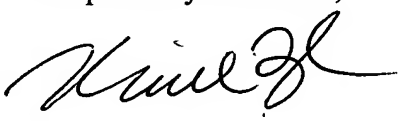
Given the information in the art at the time the present application was filed, and the known fragility of isolated human ES cells, those skilled in the art would not have been motivated to apply the methodology of Vajta et al. to hES cells, or at least would not have had a reasonable expectation that the methodology of Vajta et al. could be successfully applied to hES cells.

Furthermore, successful/optimal hESC culture requires high density seeding. Thus, it would have been reasonable to assume that high cell viability of a thawed cell sample would be necessary for hESC recovery following freezing. Since it was generally known that vitrification results in a low level of post thaw cell viability (~1%), those skilled in the art would not have reasonably expected that vitrification would provide for successful hESC recovery post thaw.

Accordingly, Applicants respectfully submit that the successful results achieved by the claimed methods of vitrification of hES cells are unexpected. Accordingly, the present claims are not only novel, but also unobvious in view of the prior art.

In view of the foregoing amendment and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,



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MODERN TRENDS

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Vitrification can be more favorable than slow cooling

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Objective: Cryopreservation of embryos and oocytes has become an essential service for infertility treatment. The clinical application of this technology should ensure optimal survival of the embryos and oocytes that are stored and subsequently thawed for transfer. The aim of this review is to compare the widely employed slow cooling procedures with vitrification to evaluate and recommend the more effective and safer procedure.

Design: The review is mainly based on a comparison of the principles, procedures, and results reported in the literature. A historical description of vitrification and personal experiences with this technology are also included.

Setting: University-based hospitals and private clinics that treat infertility and have published information on cryopreservation.

Patient(s): Women being treated for infertility and reproductive technology clinics.

Intervention(s): The application of slow cooling involving a range of cooling rates is compared with vitrification using rapid and ultrarapid cooling in simple containers. The purpose of both techniques is the induction of a glasslike state in cells to protect them from damage by ice crystals. The early development of vitrification involved the use of long pre-equilibration procedures. Improved methods resulted from the use of mixtures of penetrating and nonpenetrating solutes that are not toxic and a range of cooling rates.

Main Outcome Measure(s): Reported number of pregnancies established after transfer of embryos that were cryopreserved by vitrification, or transfer of embryos derived from vitrified oocytes.

Result(s): Both slow cooling and vitrification procedures have resulted in the successful cryopreservation of human embryos and oocytes. Both procedures have resulted in healthy births, although the slow cooling of oocytes gives very low success rates. Vitrification is a promising novel technique in assisted reproductive technology, but comparative success rates are yet to be established.

Conclusion(s): Vitrification is a simple procedure that requires less time and is likely to become safer and more cost effective than slow cooling. (Fertil Steril® 2002;78:449–54. ©2002 by American Society for Reproductive Medicine.)

Key Words: Cryobiology, embryo, oocyte, vitrification, slow cooling

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Cryopreservation of embryos and oocytes has become an integral part of improving the success rate of infertility treatment. The clinics that employ this technology should, therefore, employ those cryopreservation procedures that are likely to be embryo friendly and oocyte friendly. This implies choosing low-temperature technology that is likely to minimize damage and enhance survival rates of the living material that is stored for infertility treatment. Our review compares slow cooling with vitrification, including some background to this procedure, to evaluate which is likely to be more suitable for assisted reproductive technology (ART) as currently practiced.

One of the primary concerns in ART is to minimize the duration of exposure of gametes and embryos to potentially damaging conditions. To deal with this problem, different types of supporting media were developed, and special equipment such as controlled gas incubators, warm stages, and humidity chambers for microscopes were designed. Among the important advances in human ART is the development of protocols for effective cryopreservation of embryos and oocytes. In relation to such considerations, one of the central issues is whether slow-cooling or rapid-cooling protocols that have been employed in human IVF satisfy the fundamental principle of minimizing damage during cryostorage.

DEFINITION AND HISTORY OF VITRIFICATION

Vitrification is a process that produces a glasslike solidification of living cells that completely avoids ice crystal formation during cooling. Equally important, the vitrification process completely avoids ice crystal formation in cryopreserved cells during warming to recover the cells for biological applications. Retrospectively, the phenomenon of vitrification was first investigated and described at the turn of the 19th century (1). The founder of cryobiology, Luyet, recognized the potential of achieving an ice-free, structurally arrested state for cryopreservation >60 years ago and described it in his classical studies (2, 3). Subsequently, it was generally recognized that supporting solutions for vitrification would be better for the preservation of living cells and tissues than would solutions that crystallize and hence damage cells during cooling and warming.

However, during the early studies it was not clear how an effective vitrification procedure could be established. Vitrification requires very high cooling rates and, at the same time, protection from potentially toxic chemicals. Whereas during the early investigations the majority of these problems were not resolved, a decision was made to use stepwise procedures that replaced the vitrification approach for a long time. Many mathematical models were employed and many elegant slow-cooling protocols were designed to make these stepwise procedures effective for the cryopreservation of different types of cells and tissues.

It should be emphasized that as a result of misunderstanding the nature of slow cooling, this process is sometimes referred to as a "freezing." Freezing assumes cooling and solidification with formation of ice. In fact, ice forms in a large volume of the container that is usually employed for loading the cells or tissues in cryopreservation procedures. However, ice should never be allowed to appear and grow inside the cells or tissue as this leads to damage and death of the living system. It is important to realize that the final goal of both *slow cooling* and *vitrification* is the same: to induce a glasslike solidification inside cells to protect them from damage by ice crystals at all stages of cryopreservation. The similarity of both approaches will be described in detail later in this review.

Nevertheless, the vitrification of moderately concentrated aqueous solutions remained a subject of only academic interest until the last few decades. More recently, proposals were made to use a combination of high concentrations of solutes and rapid cooling rates to allow vitrification throughout the biological system (4–6). If used correctly, this approach would avoid both intracellular and extracellular ice formations.

VITRIFICATION IN EMBRYOLOGY

Principles

The procedure for successful cryopreservation by vitrification of mammalian embryos and oocytes, including human, has been the subject of intense research over many years. In 1985, Rall and Fahy (7) showed for the first time that murine embryos could be successfully cryopreserved by vitrification. The initial solution (VS1) was very toxic, but those investigators found ways to overcome this problem.

To slow down or restrict permeation and to reduce toxicity of the cryoprotectant solutions to embryos, equilibration of the cryoprotectant in exposed embryos was performed in a cold room. In these older studies, some elements of slow-cooling procedures, rather than modern vitrification protocols, were used. Thus, embryos were partially pre-equilibrated and exposed to a vitrification solution at a reduced temperature (4°C) (7, 8). Subsequently, embryos were immersed into liquid nitrogen after a gradual increase of the concentration of cryoprotectants. In some cases, the overall duration of embryo exposure was up to 50–60 minutes (8).

We can now compare this early vitrification protocol with a typical slow-cooling procedure. During slow cooling, embryos are usually pre-equilibrated in either 1.5 M dimethyl sulfoxide or 1.5 M propylene glycol at room temperature. The concentration of cryoprotectants is then gradually increased inside and outside the embryos during several steps over a 2-hour period. When the concentration of cryoprotectant was high enough to support glasslike solidification of the cells and the outside solution, at temperatures approaching −33°C to −40°C, the cells could then be rapidly exposed to much lower temperatures such as −150°C or lower. Nowadays, we can call the Rall and Fahy studies (7) a bridge between vitrification and slow cooling.

During the early era, the changes in cell volume were noticed and characterized. The moment when the cytoplasm becomes sufficiently concentrated and is capable of being vitrified upon cooling to a low temperature was recognized as the key moment of the procedure (8).

The initial solutions were more toxic (7, 9, 10) to embryos than are those that are currently used, but all included substantial concentrations of penetrating cryoprotectants. It was essential to formulate a nontoxic and efficient vitrification solution for vitrification. Several groups undertook a systematic and an extensive investigation involving a number of combinations of cryoprotectants, including sugars and polymers, in an effort to identify the ideal and least toxic vitrification solution (11–14).

Such studies led to the establishment of modern solutions that are more effective because they include nonpenetrating additives (13–19). Using the improved vitrification solutions and technology, human oocytes and embryos can be pre-

TABLE 1

A comparison of vitrification with slow-cooling procedures.

Accessibility and regulation	Vitrification	Slow cooling
Can be observed	yes	no
Can be analyzed	yes	no
Interaction with the oocyte or embryo	yes	no
Control of solute penetration	yes	no
Control of dehydration rate	yes	no
Maintenance of physiological temperature during equilibration procedure	yes	no
Duration out of incubator	~10 min	~3 h
Prolonged temperature shock	no	yes
Interference with oocyte or embryo	low	high
Fracture of zona pellucida	no	possible
Capture by growing ice crystals	no	possible

Kuleshova. Vitrification of oocytes and embryos. Fertil Steril 2002.

equilibrated and introduced into vitrification solutions at room temperatures (12, 19, 20) or even at physiological temperatures, the latter possibility being particularly important for mammalian oocytes of certain species. A detailed comparison of the advantages and disadvantages of slow cooling and vitrification is shown in Table 1.

Practical Aspects

Vitrification procedures are widely used for cryopreservation of ovine, equine, murine, rabbit, bovine, and porcine embryos at all stages of embryonic development, including blastocyst stages (15, 17, 18, 21–27). Human embryos have been successfully vitrified in ethylene glycol-trehalose based solutions since the mid-1990s (28). Recently, vitrification of day 2 and 3 embryos in 250-μL straws, and their subsequent thawing and transfer, has resulted in the birth of healthy twin babies (12). This protocol for human embryos was based on the assessment of suitable conditions for eight-cell mouse embryos.

Another group carrying out a trial of vitrification of morula and blastocyst stage human embryos has reported several pregnancies using the same approach (29). Employment of another vitrification solution, previously evaluated for animal embryos, for human blastocyst has also led to an advanced-stage pregnancy (30). A further novel ultrarapid technique has been developed using animal embryos and was subsequently adopted successfully in vitro on human blastocysts (31, 32). Furthermore, one of the first cases, if not the first, of live birth after vitrification of embryos at advanced stages of embryonic development, such as blastocyst stages, was reported in 2001. This success was achieved by employment of conventional 250-μL straws and rapid cooling in nitrogen vapor (33).

Oocyte cryopreservation is still at an early developmental stage. Historically, a slow-cooling technique, appropriate for human embryos, has been implemented to the preservation of human oocytes (34). Further development of slow-cooling procedures in the mid-1990s seemed to be promising at the beginning; however, this was not capable of abolishing the expected difficulties faced by all slow-cooling procedures (35–37). Slow cooling of mature human oocytes was studied extensively and is comprehensively understood (35–38). The introduction of an alternative cryoprotectant and intracytoplasmic sperm injection also have had marginal effects on the overall success rate.

Outcomes of the most consistent and long-term study resulted in only nine pregnancies (seven singletons and two twins) and the birth of 11 babies from transfers after the insemination of 1,502 thawed eggs, giving a success rate of 0.7% (39). Another group also conducted an elegant study on slow-cooled human oocytes and reported viable pregnancies involving several advanced fetuses with a success rate of 1% (40).

Fortunately, it has been suggested that the vitrification approach may also be appropriate for human oocytes. It has been determined that a stepwise pre-equilibration procedure, in which the amount of penetrating cryoprotectant is gradually increased, and a dilution procedure, based on prevention of unwelcome expansion or prolonged dehydration of cells, are very effective for human oocytes (41). As a result of these studies, one of us reported for the first time the birth of a healthy baby girl in June 1999 after transfer of an embryo derived from vitrified mature oocytes (41). Later, three patients achieved singleton pregnancies after transfer of embryos derived from vitrified metaphase II oocytes in seven patients. Two women subsequently delivered healthy male infants in August and December 1999. A third patient was continuing her pregnancy at 25 weeks of gestation (42).

Previously, it was found that prolonged exposure to a temperature lower than the physiological temperature has deleterious effects on the organization of the cytoplasm, including the integrity of the meiotic spindle of human oocytes (43). It has been reported that even transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte and therefore result in chromatid nondisjunction after fertilization (44). An increased frequency of chromosome abnormalities after fertilization of frozen-thawed human oocytes was also reported (37, 45).

Oocytes at the germinal vesicle (GV) stage are characterized by extended chromosomes held within a nuclear membrane, the absence of a spindle, cortical granules that are not ready to be released, and a compact cumulus. For these reasons, it has been proposed that GV-stage oocytes are less sensitive to cryopreservation damage than are mature oocytes at the metaphase II stage. It was therefore assumed that freezing of immature oocytes may be an alternative approach to the freezing of mature oocytes. However, in early studies, slow cooling of immature oocytes using dimethyl sulfoxide gave poor results (46).

Comparative and long-term studies were carried out on slow-cooled and vitrified human oocytes over the past 3 years at Pochon CHA University, South Korea (19, 47, 48). Although the fertilization and development rates of mature oocytes, either untreated (91% and 94%, respectively) or treated with a cryopreservation solution such as 1,2-propanediol (81% and 88%, respectively) were high, low fertilization (42.9%) and development rates (16.7%) were reported after slow cooling of immature human oocytes using the above-mentioned cryoprotectant. Oocyte cryopreservation capacity improved dramatically by replacement of the traditional freezing methods with vitrification. In their recent report, the majority of embryos derived from vitrified oocytes developed to two-cell (71%–100%), four-cell (71%–93%), eight-cell (46%–71%), and blastocyst (29%–36%) stages (48).

Current Information About Vitrification Methods and Future Trends

The concept that living systems could be cooled so quickly that ice would not have time to form was proposed >60 years ago (2, 3), and this proposal seems more realistic now. During the advances that took place, a variety of new techniques and types of holders that enhance the cryopreservation of mammalian oocytes and embryos were developed. These include immersion in microcapillary straws (permit a cooling rate of 2,500°C/min), on film of a nylon loop (31, 32), in nonsterile heat-softened and pulled straws (cooling rate of 20,000°C/min) (49), duck-loading tips, and aluminum foil (50). Electron microscope gold or copper grids (51) can also be used very successfully as oocyte containers during cooling and warming (19, 48, 51).

Nevertheless, the latest and most successfully applied approach has introduced a problem related to storage. It is well known that many viruses may survive direct exposure to liquid nitrogen and therefore could potentially cause contamination (52, 53). Development of new embryo culture systems that employ serum-free media may reduce or eliminate sources of contamination (54). It was determined over several years in our studies that keeping the 250-μL straw containing the embryos inside a second protective container (500-μL straw) throughout vitrification, storage, and warming would be effective for animal and human embryo cryopreservation (55) (Kuleshova LL, Odawara Y, unpublished observations).

This is a very simple strategy for reducing or eliminating the potential contamination risk. The novel set of solutions and the modified straw-in-straw procedure does not compromise embryo viability in humans. The survival and development rates of vitrified day 3 and day 4 human embryos, as assessed in vitro, were not significantly different from those in controls. Therefore a cooling rate of 400°C/min (straw in straw) is effective for human embryo vitrification.

However the critical requirement was to combine a high cooling rate with a safe procedure. Sometimes, a very simple solution can be found, for example, vitrification of human oocytes inside a commercially available sterile stripper tip that combined extremely high cooling rates with a greatly reduced risk of losing specimens (Kuleshova LL, Otani T, unpublished observations). The sterile stripper tips for removing a cumulus have a diameter smaller than that of other containers used for vitrification, allowing a rate of cooling approaching that of the rate in electron microscopic grids. Uniform adjustment of the pipette and convenient manipulation either during stepwise equilibration procedures or under liquid nitrogen indicate that the sterile stripper tips can be considered as tools for the cryopreservation of human oocytes and embryos. Moreover, the possibility of problems with the other containers, such as the bending of aluminium foil or metal grids; or even in rare cases the cracking of a heated, softened, and then pulled straw during storage; suggests that the proposed new container may have an advantage over those that are currently in use.

Recently it was found that human mature oocytes could be successfully vitrified by immersion in liquid nitrogen in conventional 250-μL straws (56). Our preliminary data have also shown that cooling at a rate of 400°C/min was also appropriate for human immature oocytes and blastocysts. It was shown that employment of conventional 250-μL straws and rapid cooling in nitrogen vapors, which gives a moderately low cooling rate such as 120°C/min, was effective for vitrification of human embryos at all stages of development including blastocyst stages (12, 33).

It seems that employment of ultrarapid techniques or the standard rapid technique described here should make little difference for successful vitrification of human oocytes and embryos. It is predicted that the effects on oocytes and embryos would be more distinct when much greater cooling rates than are presently employed will become available. Further investigations are required to evaluate this in practice.

In conclusion, an answer to the question posed in the title of this article is that the use of slow-cooling protocols for the cryopreservation of embryos and oocytes breaches the principle that gametes and embryos should not be exposed to unphysiological conditions for longer than necessary before their storage at low temperatures.

We can also conclude that the history of cryopreservation is based on a background of the development of vitrification in cell biology. The initial theoretical postulates and principles on vitrification were replaced by a long period of slow-cooling protocols that were believed to be suitable for low-temperature preservation of cells and tissues. Now the field of cryobiology has progressed by returning to vitrification protocols.

The early, long vitrification protocols have been replaced by very short and more effective procedures, and it is envis-

aged that the time has come when vitrification of human oocytes and embryos will be used more widely.

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Technical Article

A simple and efficient cryopreservation method for primate embryonic stem cells

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ABSTRACT Human embryonic stem (ES) cells have the potential to differentiate into all cell types. As these cells may be able to provide an unlimited cell source for transplantation therapies, it is necessary to establish reliable methods for their handling and manipulation, including human ES cell cryopreservation. Here, we report the development of a simple and efficient cryopreservation method for primate ES cell lines using vitrification in conventional cryovials. Using standard slow-rate cooling methods, the cryopreservation efficiency for cynomolgus monkey ES cell lines was approximately 0.4%, while that for a human ES cell line was virtually 0%. Primate ES cell lines, however, were successfully cryopreserved by the present vitrification method using conventional cryovials yielding a survival rate of about 6.5% for monkey ES cells and 12.2% for human ES cells. Vitrified ES cells quickly recovered after thawing and exhibited a morphology indistinguishable from non-vitrified cells. In addition, they retained a normal karyotype and continued to express ES cell markers after thawing. Thus, our vitrification ES cell cryopreservation method expands the utility of primate ES cells for various research and clinical purposes.

KEY WORDS: *primate embryonic stem cell, human embryonic stem cell, cryopreservation, vitrification*

Introduction

Embryonic stem (ES) cell lines are derived from the inner cell mass of blastocysts and can proliferate indefinitely *in vitro*. The defining feature of these cells is their potency to differentiate into a variety cell types of all three embryonic germ layers: the ectoderm, mesoderm and endoderm (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Thus these characteristics make ES cell lines valuable for research in developmental biology and transplantation therapy (Keller and Snodgrass, 1999). Non-human primate ES cells, such as cynomolgus monkey ES cells, have been extensively used as a model system in preclinical experiments to examine the clinical applications of human ES cells (Thomson *et al.*, 1995 and 1996; Suemori *et al.*, 2001). The sensitivity of both human and monkey ES cells to cryopreservation, however, has severely limited the utility of these cells (Reubinoff *et al.*, 2001). For example, difficulties in the distribution of cell lines have made the reproduction of experiments using primate ES cells problematic. Therefore, it is necessary to establish reliable methods for the cryopreservation of primate ES cells. Slow-rate cooling methods using dimethylsulfoxide (DMSO) as a cryoprotectant are effective for a wide variety of cell lines, including mouse ES cell lines. In most protocols, cells are suspended in freezing medium containing DMSO at 5-20%, transferred into glass or plastic cryovials and then frozen by cooling at 1.0 to 2.0°C/ min. The ability to freeze the majority of cell lines with common laboratory equipment to yield apparatus with satisfactory survival rates after thawing has made these protocols widely used. Such conventional methods, however, are not applicable to primate ES cell lines, as these cells die immediately after thawing. Although the reason why primate ES cells cannot survive the freeze-thaw process is not clear, damage by ice crystal formation in the cytoplasm during the freezing process is one of the possible causes of cell death (Dobrinsky, 1996). A rapid cooling method, vitrification, has been used for the cryopreservation of fertilized eggs or embryos of several mammalian species, including human (Rall and Fahy, 1985; Kuleshova *et*

Abbreviations used in this paper: DAP medium, DMSO+Acetamide+Propylene glycol; DES medium, DMSO+Ethylene glycol+Sucrose; DMSO, dimethylsulfoxide; EFS medium, Ethylene glycol+Ficoll+Sucrose; ES, embryonic stem; MEF, mouse embryonic fibroblast; OPS, opened pulled straws.

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al., 1999; Dobrinsky, 2002), to prevent ice crystal formation (Luiet, 1937). Therefore, we postulated that primate ES cells may be effectively cryopreserved by vitrification. Although a report demonstrating that human ES cells could be successfully cryopreserved by vitrification (Reubinoff *et al.*, 2001) supports this hypothesis, the protocol was both labor consuming and impractical on a large scale, as it requires a special device of open pulled straws (OPS) and the manual picking-up of individual stem cell colonies. In addition, this technique encourages potential contamination with pathogenic agents (Gorman and Patterson, 1995) due to the direct exposure of ES cell suspensions to liquid nitrogen filled in OPS. Such risks must be avoided for the potential clinical use of human ES cells. Therefore, we developed a novel cryopreservation method for primate ES cells that is simple and efficient on a large scale.

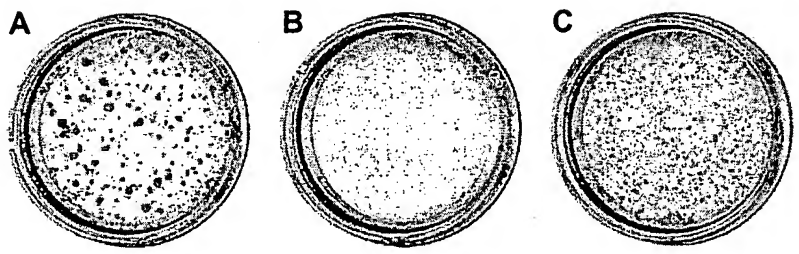

Fig. 2. Typical morphology of CMK6 ES cell colonies after subculturing, slow-cooling and vitrification. (A) *A control plate prepared by passaging as described in Experimental Procedures. ES cell colonies at five days after cryopreservation by* **(B)** *the slow-cooling and* **(C)** *the vitrification method.*

Experimental Procedures

ES cell culture

The cynomolgus monkey ES cell lines CMK6 and CMK9 were established as described (Suemori *et al.*, 2001) and cultured on mitomycin C-treated mouse embryonic fibroblast (MEF) feeder cell layers. Monkey ES were cultured in CMK medium consisting of DMEM/F-12 supplemented with 0.1 mM 2-mercaptoethanol, 20% KSR (Invitrogen Corp., CA, USA), 2 mM L-glutamine and 1% MEM nonessential amino acids. The establishment and culture of human ES cell line, KhES-1, are described elsewhere (H. Suemori, in preparation). Briefly, human ES cells were cultured in CMK medium supplemented with 5 ng/ml recombinant human FGF-2 (Upstate, VA, USA) on MEF feeder cells and the ES cells were subcultured every 3-4 days by enzymatic dissociation using a dissociation solution consisting of 0.25 % trypsin, 1 mg/ml collagenase, 1 mM $CaCl_2$ and 20% KSR in PBS. The establishment and use of human ES cell lines was performed in conformity with «The Guidelines for Derivation and Utilization of Human Embryonic Stem Cells (2001)» of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

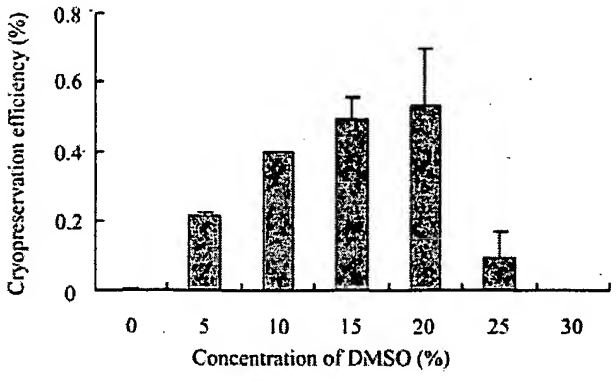

Fig. 1. Cryopreservation efficiency of CMK6 ES cells by slow cooling method. *CMK6 ES cells were frozen in freezing media containing 0 to 30% DMSO. The number of ES cell colonies was counted at 6 days after thawing and compared to the control plate.*

Slow-rate cooling method

Confluent cultures of ES cells in 100 mm plates were dissociated using in dissociation solution. ES cell suspensions were divided into three centrifugation tubes containing equal cell numbers and one tenth of each suspension was replated onto feeder layers, cultured and used as a control for calculating cell viability after thawing. After centrifugation, the cell pellets were resuspended in freezing medium containing 10% DMSO in CMK medium and transferred to 1 ml cryovials (Nalge Nunc International, NY, USA, SYSTEM 100 Cryogenic Vial). The cryovials were placed into a Nalgene™ Cryo1°C Freezing Container (Nalge Nunc International). ES cells were stored in a –80°C freezer overnight, then transferred to a –150°C freezer until thawing.

The ES cells were thawed at 37°C in a water bath with gentle shaking and the ES cell suspension was transferred into a centrifugation tube filled with 10 ml CMK medium. After centrifugation, the supernatant was removed and the ES cell pellets were resuspended in 2 ml CMK medium. The cells were then plated in 6 well culture plates or 35 mm culture dishes with a feeder layer, cultured for 6 days and then evaluated for their survival rates.

Vitrification methods

ES cells were prepared as described above. Three kinds of freezing media, DAP, DES and EFS40, were examined in our simple vitrification method. The composition of each freezing medium was as follows. DAP: 2 M DMSO, 1 M acetamide and 3 M propylene glycol in CMK medium; DES: 20% DMSO, 20% ethylene glycol and 0.5 M sucrose in CMK medium and EFS40: 40% ethylene glycol, 18% ficoll 70,000 MW and 0.3 M sucrose in CMK medium. After resuspending the ES cells in 200 µl of freezing medium, samples were transferred to cryovials and immediately soaked in liquid nitrogen so that 2/3 of the vial from the bottom up was immersed, thereby avoiding contamination by the coolant. The cryovials were then stored in a –150°C freezer until thawing.

To thaw the vitrified ES cells, medium pre-warmed to 37°C was added to the vials. The cells were thawed quickly by pipetting and after centrifugation, ES cells were plated as described for the cells subjected to slow cooling as described above. The thawed cells were cultured for 4 days and evaluated for their survival rates.

Assessment of ES cell growth after cryopreservation

To evaluate the survival rate after cryopreservation, the efficiency of the slow-rate cooling method was assessed by counting the number of ES cell colonies, while that of the vitrification method

was assessed by counting ES cell numbers. The number of ES cells or ES cell colonies was counted and compared to that of the control plates prepared as described above.

Histochemical analysis of recultured ES cells

ES cells were fixed in 4% paraformaldehyde for 10 min. Alkaline phosphatase activity was detected with Vector Red Alkaline Phosphatase Substrate Kit I (Vector laboratories, CA, USA). The expression of SSEA-4 and TRA1-60 was detected by immunostaining using specific antibodies (CHEMICON International Inc., CA, USA) diluted at 1:100. After 2 hours of incubation, antibody staining was visualized with an HRP-labeled secondary antibody and diaminobenzidine.

Teratoma formation

Several passages after thawing, ES cells were dissociated. Approximately $1x10^7$ ES cells were subcutaneously injected into SCID mice. Teratomas were formed within 5 to 8 weeks after injection. Teratomas were removed by dissection, fixed in 4% formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin.

Results and Discussion

Slow-rate freezing of monkey ES cells

For biological research and medical applications, it is necessary to develop an efficient cryopreservation method for the primate ES cell lines, CMK6 and CMK9. We therefore have attempted to establish an efficient and simple cryopreservation protocol applicable to primate ES cells. We first examined the efficiency of the slow-rate cooling method using freezing medium containing 10% DMSO for primate ES cells. It was necessary to determine the cell number after thawing to accurately evaluate the survival rates. However, this was impossible because of extremely poor recovery associated with this method (Fig. 2B). Thus, we estimate the survival rates by counting the number of colonies. After thawing, very few CMK6 cells remained alive, resulting in a cryopreservation efficiency of 0.4% compared to that for passaging (Fig. 1). To optimize the cryopreservation medium, we tested several concentrations of DMSO ranging from 0-30%. Although 20% DMSO proved to be most effective for slow cooling, the efficiency was only increased to 0.6%. We also examined a range of commercially available freezing media and tested the effect of additional components such as methylcellulose (Ohno *et al.*, 1988) on the freezing efficiency. However the cell viability after thawing scarcely improved.

From these results, primate ES cells were found to exhibit low survival rates after cryopreservation using the slow-rate cooling method. Two weeks or more of expansion was required for undifferentiated stem cells to proliferate enough for subculturing. Thus, this time consuming and unreliable protocol is not practical for laboratory and clinical applications of primate ES cells.

Vitrification freezing of monkey ES cells

Vitrification is an alternative way to cryopreserve a variety of cell types, including mammalian fertilized eggs or embryos (Rall and Fahy, 1985; Kuleshova *et al.*, 1999; Dobrinsky, 2002). Although vitrification of human ES cells has been previously attempted (Reubinoff *et al.*, 2001), the potential contamination risks (Gorman and Patterson, 1995) and limited utility of this method has made it necessary to develop more efficient, reliable and simple vitrification methods.

As cryovials are the most commonly used cryopreservation containers for cultured cells, we replaced the OPSs used in this previously described method to include these vials. We also compared the efficacy of three freezing media, DES, DAP and EFS40, to optimize the vitrification protocol using cryovials. While DES (DMSO, Ethylene glycol and Sucrose) was used for the vitrification of human ES cells using OPS (Reubinoff *et al.*, 2001), DAP (DMSO, Acetamide and Propylene glycol) is used for the cryopreservation of mammalian oocytes and embryos (Nakagata *et al.*, 1993). EFS (Ethylene glycol, Ficoll and Sucrose) is also used for the cryopreservation of mammalian embryos in glass capillaries (Kasai *et al.*, 1990; Nagy *et al.*, 2003). The concentration of ethylene glycol in EFS medium varies widely among

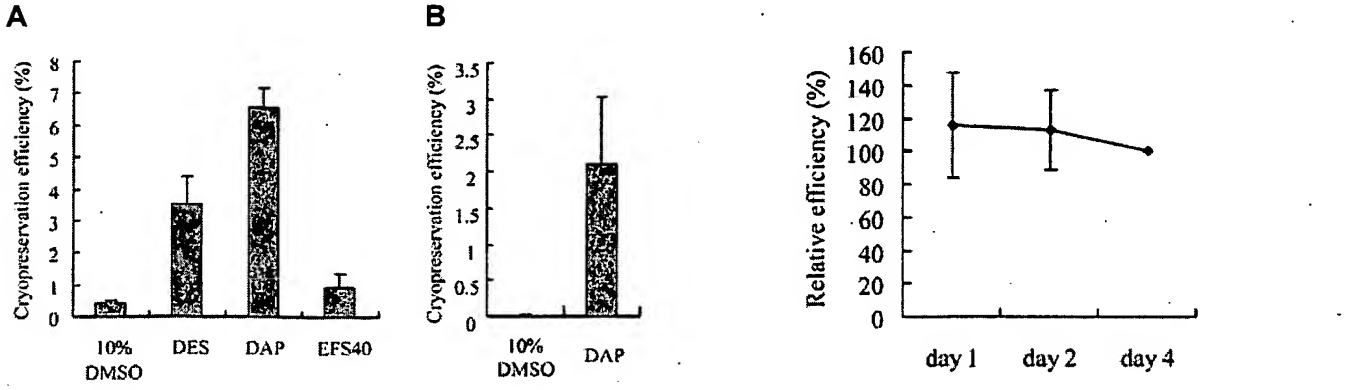

Fig. 3 (Left). Efficiency of cryopreservation by vitrification. (A) *The average survival rate of CMK6 ES cells after vitrification using DES, DAP and EFS40 freezing media was estimated by counting cell numbers at four days after thawing. The efficiency of the slow-cooling method using 10% DMSO is also shown.* **(B)** *Cryopreservation efficiency for CMK9 cell lines was examined as in (A).*

Fig. 4 (Right). Recovery of ES cells after cryopreservation. *The survival rate of CMK6 ES cells was evaluated 1, 2 and 4 days after thawing from vitrification using DAP. The cryopreservation efficiencies for day 1 and day 2 relative to day 4 are shown.*

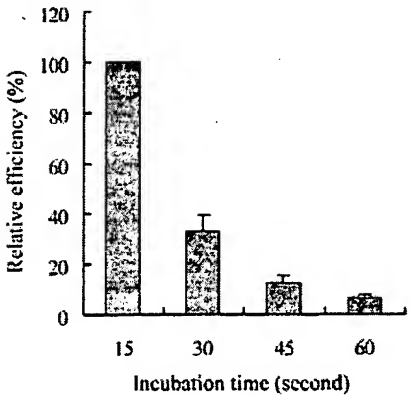

Fig. 5. Effect of incubation time in DAP during vitrification on the cryopreservation efficiency of CMK6 ES cells. *ES cells were incubated in DAP for 15, 30, 45 and 60 seconds before freezing. The cryopreservation efficiency was then evaluated 4 days after thawing and it was found that the efficiency rapidly decreased in accordance with the increase in incubation time.*

protocols. We chose the EFS40 medium containing 40% v/v ethylene glycol, as this medium is widely used for mouse embryo cryopreservation. Since the vitrification methods yielded higher cell recovery rates than did the slow-rate freezing methods as shown in Fig. 2, we were able to accurately evaluate the survival rates by determining the cell number instead of the colony number. Among the vitrification freezing media examined, DAP exhibited the highest recovery rate of 6.5% (Fig. 3A), nearly twice as high as the efficiency using DES (3.5%). EFS40 displayed a very low efficiency of 1.0%.

The cryopreservation efficiency was also examined using another cynomolgus monkey ES cell line, CMK9. The viability of vitrified CMK9 cells was about 2.1% (Fig. 3B). It was very low compared with that of CMK6 cells, which was about 6.5%. Such differences may represent a higher sensitivity of CMK9 cells to mechanical damage as suggested by previous experiments (Furuya *et al.*, 2003).

We next tried to optimize this vitrification method using DAP. We examined a range of DAP concentrations from 0.25-1.5 fold of the initially tested concentration. The original concentration of DAP proved to be most effective. We attempted to improve the freezing efficiency by varying the ratios of the DAP components. Although we tested concentrations of DMSO ranging from 15-30% and propylene glycol ranging from 15-30%, we found little improvement in efficiency compared to the original DAP medium (data not shown).

We further analyzed the cryopreservation efficiency for CMK6 ES cells by vitrification using DAP immediately after thawing. As shown in Fig. 4, the cells exhibited rapid recovery from cryopreservation and exhibited similar survival rates at 1, 2 and 4 days after thawing.

In a subset of previously reported vitrification protocols, pretreatment with a less hypertonic medium than the freezing medium improved the efficiency of cryopreservation (Rall and Fahy, 1985). For the OPS vitrification method, ES cells were exposed to 1 M DMSO for 1 min before freezing. Therefore, we pretreated CMK6 cells with 1 M DMSO for 1 min before vitrification in DAP. This pretreatment, however, did not significantly alter the cryopreservation efficiency.

As vitrification freezing media contain high concentrations of cryoprotectants, these media are toxic to ES cells. Therefore, we examined the effect of the duration of time the cells spent suspended in the freezing medium before soaking in liquid nitrogen on cell survival after thawing. The survival rates decreased with increased holding time before freezing (Fig. 5); when the holding time was longer than 1 min, cryopreservation efficiency was lower than 0.5 %. Therefore, EFS40 did not permit good recovery rates (Fig. 3A), probably because it is too viscous to handle rapidly. A

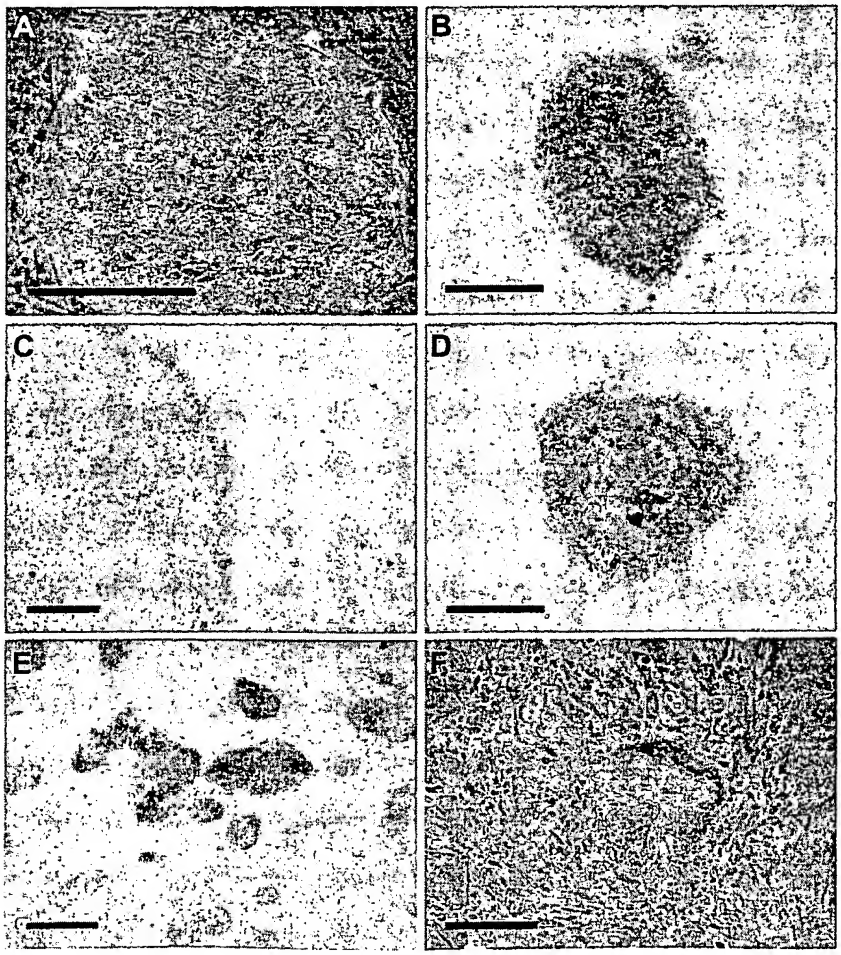

Fig. 6. Morphology and stem cell marker expression in CMK6 ES cells after cryopreservation. *A phase contrast microscopic image of vitrified CMK6 at 3 days after thawing* **(A)** *and vitrified CMK6 cells examined for alkaline phosphatase activity* **(B, E-F)**. *Immunocytochemistry of vitrified CMK6 detected the expression of SSEA-4* **(C)** *and TRA1-60* **(D)**. *A low power view of vitrified ES cells at 5 days stained for alkaline phosphatase activity showing that most colonies retained their undifferentiated state* **(E)**. *A typical differentiating colony* **(F)**. *Scale bars, 20 μm (A-D, F); 50 μm (E).*

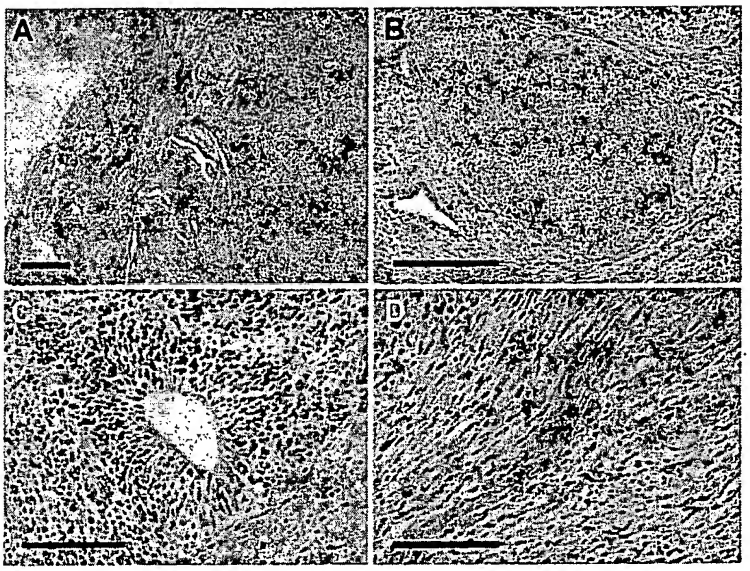

Fig. 7. Teratoma formation by vitrified CMK6 ES cells. *The gross appearance* **(A)** *and higher magnification views* **(B-D)** *of a H & E-stained section of a teratoma produced from vitrified CMK6 ES cells. Cells expressing cartilage* **(B)**, *neuroepithelium* **(C)** *and muscle* **(D)** *phenotypes were identified. Scale bars, 20 μm (A,B); 10 μm (C, D).*

holding time of approximately 15 s is thought to be the minimal handling time required for suspending cells, transferring to vials and freezing. Then, the greater time needed to manipulate the cells until freezing leads to increased cell death due to cryoprotectant toxicity.

We also examined the effect of the cell concentration in the cryovials on the survival rate. CMK6 cells were subjected to our vitrification protocol over a wide rage of cell concentrations, from $5x10^5$ to $1x10^7$ cells/vial. Regardless of the cell density, ES cells were successfully recovered at similar efficiencies. This result suggested that primate ES cells can be cryopreserved in cryovials for any culture scale, from 35 mm dishes to 100 mm or larger. The independence of the efficiency from culture size makes this protocol advantageous for a variety of clinical and research applications using primate ES cells.

Reubinoff *et al.* demonstrated that 100% of the human ES cell colonies could be recovered after cryopreservation using the OPS vitrification method (Reubinoff *et al.*, 2001). This difference in efficiency from our protocol was likely caused by differences in the vitrification protocols and assay systems. They evaluated the recovery efficiency by comparing colony numbers before and after vitrification, but he actual cell survival rate was not presented. In addition, more than 70% of the colonies exhibited a differentiated phenotype at 7 days after thawing by their OPS vitrification method. Using our culture system, spontaneous differentiation of primate ES cells was rarely observed (Fig. 6 E,F); less than 1% of the colonies exhibited differentiated morphology during routine maintenance. The rate of occurrence of differentiated colonies increased to 3% after vitrification and this might have been caused by a relatively higher tolerance of differentiated cells to damage induced by the vitrification processes. Thus, our vitrification method induces differentiation at a lower rate.

Cell characteristics after cryopreservation

Our vitrification method using DAP freezing medium permitted the rapid recovery of ES cells after cryopreservation. Usually, approximately two weeks are required following cryopreservation by slow-rate cooling for human ES cells to grow to the cell number present at the time of freezing. By our vitrification method, this number of cynomolgus monkey ES cells was recovered within about 4 days. The thawed ES cells tend to form small colonies compared with control ES cells probably because of additional dissociation caused by handling during freezing/thawing and damage induced by the preservation process (Fig. 2). Vitrified ES cells recovered very quickly and could be maintained for more than one month without any signs of differentiation or changes in their growth properties. Since human ES cells tend to differentiate after thawing (Reubinoff *et al.*, 2001), our vitrification method may be an effective way to minimize this side effects. Such quick and safe recovery of ES cells also reduces the time and cost of experiments requiring cryopreservation from those using conventional slow-rate cooling methods.

Following the culture of vitrified ES cells, we assessed their stem cell marker expression (Suemori *et al.*, 2001). Thawed cynomolgus monkey ES cells continued to exhibit the alkaline phosphatase activity characteristic of ES cells and also expressed the stem cell markers SSEA-4 and TRA1-60 (Fig. 6). We also analyzed the karyotype of CMK6 cells post-vitrification, demonstrating that these ES cells retained a normal karyotype of 40+ XY chromosomes (data not shown). To examine differentiation potential of vitrified ES cells, we examined the formation of teratomas by injecting ES cells after vitrification into SCID mice. Teratoma formation was observed in two independent experiments. Histological analysis of isolated teratomas revealed that the vitrified ES cells differentiated into a variety of cell types, including cartilage, neuroepithelium and muscle (Fig. 7). Thus, the vitrified ES cells retained their pluripotency.

Human ES cell cryopreservation

We examined the potential application of this vitrification method to cryopreservation of a human ES cell line, KhES-1. We could not recover any viable cells after cryopreservation by the slow-rate cooling method using 10% DMSO (Fig. 8). In contrast, human ES cells both survived and continued to grow after vitrification in DAP. The cryopreservation efficiency of KhES-1 was approximately 12.2%. As seen for monkey ES cells after vitrification, the vitrified human ES cells were indistinguishable from non-vitrified ES cells.

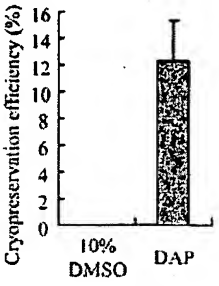

Fig. 8. Cryopreservation efficiency of a human ES cell line. *The survival rates of a human ES cell line, KhES-1, after cryopreservation by the slow-cooling method using 10% DMSO and by the vitrification method using DAP.*

In conclusion, we have developed a novel cryopreservation method for primate ES cell lines, including human ES cell lines. This vitrification method provides the simple, efficient and reliable cryopreservation of primate ES cell lines. Such a system will be exceedingly helpful for experimental research and medical applications.

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Cryopreservation of human embryonic stem cells by vitrification

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Keywords: *cryopreservation* · *vitrification* · *human embryonic stem cell*

Background The efficiency of traditional cryopreservation of human embryonic stem (ES) cells is low, and there have been few attempts to prove new cryopreservation methods effective. This study was designed to evaluate the efficiency of cryopreservation of human ES cells using vitrification method.

Methods Human ES cells clumped from an identical cell line were randomly allocated to be cryopreserved by vitrification or by slow freezing. The recovery rates, the growth and differentiation potential of thawed human ES cells were compared between these two groups. The pluripotency of human ES cells after thawing was identified.

Results Eighty-one point nine percent (59/72) of human ES cell clumps were recovered after vitrification, while only 22.8% (16/70) were recovered after slow freezing ($P<0.01$). The colonies after vitrification manifested have not only faster growth but also a lower level of differentiation when compared to colonies subjected to the slow freezing protocol. However, the rates of growth and differentiation in undifferentiated colonies from both groups were identical to the rates in those of non-cryopreserved stem cells after a prolonged culture period. Passage 6 of vitrified human ES cells retained the properties of pluripotent cells, a normal karyotype and expressed the transcription factor OCT-4, stage specific expressed antigen-4 (SSEA-4) and SSEA-3. Teratoma growth of these cells demonstrated the ability to develop into all three germ layers.

Conclusions Vitrification is effective in cryopreserving human ES cells. During a prolonged culture, human ES cells retain their pluripotency after cryopreservation.

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Embryonic stem (ES) cells derived from preimplantation embryos are undifferentiated immortal cells capable of differentiation into derivatives of all three embryonic germ layers. Well-characterized ES cells have been obtained not only from rodents but also from primates in particular preimplantation embryos of humans.[1] Many reports have focused attention on the great promise of cell and tissue therapy for damaged or diseased organs resulting from, for example, spinal cord injury or degenerative diseases such as Parkinson's disease, Alzheimer's disease, etc. But the effective cryopreservation of human ES cells remains poorly discussed. Effective freezing and thawing technologies will allow effortless transfer of the cells between research centers and promote collaboration and widespread use of the cells for research and clinical applications. Currently, the survival rate of human ES cells from conventional slow freezing and rapid thawing protocols is relatively low, with most cells either dying or differentiating.[2] Reubinoff and coworkers[3] first reported the use of vitrification for the cryopreservation of human ES cells and got encouraging results. Here, the efficiency of the vitrification method is further discussed and compared with that of the conventional slow freezing method in order to encourage and facilitate the exploration of the remarkable potential of human ES cells.

METHODS

Human ES cell cultures

The human ES cell line used in this study was the cell line established in our center in November 2002 and has been named HES-1. Culture medium consisted of 80% high glucose Dulbecco's modified Eagle's medium (no pyruvate, Gibco, BRL) supplemented with 20% fetal serum (Hyclone, USA), 1 mmol/L β-mercaptoethanol (Sigma, USA), and 1% nonessential amino acid stock (Gibco, BRL).

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Colonies of HES-1 were routinely passaged by mechanical disaggregation into clumps, which were replated onto irradiated mouse embryonic fibroblasts (MEFS) in fresh medium every 7 days.

Cryopreservation of human ES cells

Human ES cell colonies from one culture dish were dissociated into clumps by mechanical methods and 142 ES cell clumps were collected. Among them, 72 cell clumps were randomly selected to be cryopreserved by the vitrification method, and the others were cryopreserved by the slow freezing method.

Vitrification protocol

Glass straws were pulled manually after heating, and then cut at the narrowest point with a razor blade, producing straws with a volume of 20—130 μl. Two vitrification solutions (VS) were used. The basic solution consisted of the routine culture medium of HES-1. The first VS (VS_1) was supplemented with 10% dimethylsulphoxide (DMSO), 10% ethylene glycol, and 0.5 mol/L sucrose. The second VS (VS_2) included 20% DMSO, 20% ethylene glycol, and 1.0 mol/L sucrose. The thawing solutions (TS) were stepwise solutions consisting of basic medium supplemented with 0.2 mol/L sucrose (TS_1), 0.1 mol/L sucrose (TS_2), and finally no sucrose. The vitrification procedures were performed at room temperature. Ten to fifteen ES cell clumps were first put into VS_1 and incubated for 1 minute, then equilibrated in VS_2 for 25 seconds, and finally, transferred into a droplet of 20 μl VS_2. The narrow end of the straw was submerged into the droplet and the clumps were loaded into the straw by capillarity. The straw was plunged into liquid nitrogen immediately after loading. All the thawing procedures were performed on a warm plate at 37℃. Three seconds after removing from liquid nitrogen, the narrow end of the straw was quickly submerged into TS_1, with the wide opening sealed with the researcher's finger. The expansion of the cool air in the straw resulting from the rise in temperature forced clumps out of the straw and into the dish. After 1 minute equilibration in TS_1, the clumps were incubated in TS_2 and then ES culture medium for 5 minutes each, before being transferred to a fresh feeder layer. When this stepwise dilution was used, DMSO was gradually removed from the cytoplasm.

Slow freezing protocol

All slow freezing procedures were performed at 0℃. Fifty to one hundred clumps were mixed with 1 ml pre-cooled freezing medium (90% ES cell culture medium and 10% DMSO) and then transferred into 2 ml cryovials. The vials were frozen at −4℃ for 1 hour, followed by −70℃ overnight. The next morning, they were transferred into liquid nitrogen for long-term preservation. At the time of thawing, the vials were placed in a 37℃ water bath. After complete thawing, the freezing medium was diluted with ES culture medium. The ES cell clump suspension was then transferred to a 15-ml tube and centrifuged at 1000 r/min for 5 minutes. The supernatant was discarded. The pellet was suspended gently in ES culture medium and plated onto a fresh feeder layer.

Assessment of ES cell growth and differentiation

The ES cells in both groups were thawed 3 days after freezing. The cell clumps that had preserved their integrity and shape were collected and plated on a fresh feeder layer for further culturing. Dispersed cells were discarded. The recovery rates (the number of collected clumps/the number of cryopreserved clumps) of both groups were accounted. All recovered ES cell clumps survived in culture. To evaluate the influence of the two cryopreservation protocols on the growth of ES cells, the longitudinal and horizontal radiuses of each colony in the two groups were measured on the 2nd, 7th, and 9th days after plating. The approximate area of each colony was calculated by using the surface area equation of an ellipse ($1/4\pi ab$, where a and b are the longitudinal and horizontal radii, respectively).[3]

Undifferentiated human ES cells have a high ratio of nucleus to cytoplasm and prominent nucleoli, and they form flat colonies of individual distinct cells. Differentiated ES cells have a large volume of cytoplasm and a relatively small nucleus. They pile up and form multicellular aggregates or vesicular structures. According to the levels of differentiation, each colony was classified as predominantly undifferentiated (differentiated cells < 30%), partially differentiated (differentiated cells 30% — 70%), and predominantly differentiated (differentiated cells > 70%). Classification of ES cell colonies was performed on the 9th day after plating in the case of both the vitrification and slow freezing groups, and results were documented and compared with each other and with non-cryopreserved ES cells on the 7th day after plating.

Identifying the pluripotence of vitrified human ES cells

At passage 6 after vitrification, ES cell surface markers including SSEA-1 (MC-480), SSEA-3 (MC-631), and SSEA-4 (MC-813-70) (related reagents from

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Iowa, USA), and alkaline phosphatase (Shanghai Biotechnology Engineering Co., China) activity of the colonies were determined by immunohistochemical tests.[1] Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to identify the expression of the stem cell transcriptional gene OCT-4. For this procedure, the primers[4] were as follows: sense, 5 '-CTTGCTGCAGAA-GTGGGTGGAGGAA-3 '; antisense, 5 '-CTGCAGTGTG-GGTTTCGGGCA-3'. Standard G-banding techniques were used for karyotyping of stem cells at passages 6 and 15.[5]

To test the capacity for differentiation *in vivo*, vitrified ES cells at passage 9 on MEFS were injected subcutaneously into mice with severe combined immunodeficiency disease (SCID). The teratoma was isolated 6 − 8 weeks after injection and subjected to histological analysis.

Statistical analysis

Student's t test and χ^2 analysis were calculated using the SPSS 10.0 statistical package. The data were presented as mean ± standard deviation (SD). A P value less than 0.05 was considered statistically significant.

RESULTS

Survival rates after thawing

In both groups, some ES cells died or separated from the clumps after freezing and thawing, leading to a recovery and survival rate of 81.9% (59/72) in the vitrified group and 22.8% (16/70) in the slow freezing group ($P<0.01$).

Growth rates after thawing

The mean areas of vitrified and slow freezing ES cells colonies on the 2nd, 7th, and 9th days of the first passage are shown in Table 1. The difference in mean area between the two groups on the 2nd day after thawing was not statistically significant (P_{d2} = 0.0642). However, the mean areas of the colonies in the vitrification group on the 7th and 9th days were larger than that in the slow freezing group. These differences were statistically significant (P_{d7} = 0.0003, P_{d9} = 0.00004).

Table 1. The mean areas of human embryonic stem cell colonies in vitrified and slow freezing groups (mean ± SD, mm²)

Time after plating	Vitrification (n=59)	Slow freezing (n=16)	P value
2nd day	0.0420±0.0062	0.0398±0.0038	>0.05
7th day	0.2254±0.0529	0.1236±0.0133	<0.05
9th day	1.0231±0.1460	0.6906±0.0719	<0.05

Differentiation of ES cell colonies

The levels of differentiation were compared between the vitrification group and the slow freezing group on the 9th day after plating and with non-cryopreserved ES cell colonies on the 7th day after plating. The non-cryopreserved and vitrified colonies were predominantly undifferentiated or partially differentiated. By contrast, most cells were differentiated in colonies from the slow freezing group. The difference between the vitrification and slow freezing groups was statistically significant (χ^2 = 19.639, $P<0.01$, Table 2).

Table 2. Differentiation of human embryonic stem cell colonies [n (%)]

Groups	n	Predominantly undifferentiated	Partially differentiated	Completely differentiated
Non-cryopreserved on 7th day	70	51 (72.8)	14 (20.0)	5 (7.1)
Slow freezing on 9th day	16	1 (6.3)	5 (31.3)	10 (62.5)
Vitrification on 9th day	59	20 (33.8)*	29 (49.1)*	10(16.9)*

* $P<0.01$ compared with the non-cryopreserved group and the slow freezing group.

Assessment of pluripotency of vitrified human ES cells

Immunohistochemical assays at passage 6 after vitrification demonstrated that the ES cells expressed cell surface markers of pluripotent cells, including SSEA-3 and SSEA-4, but did not express SSEA-1

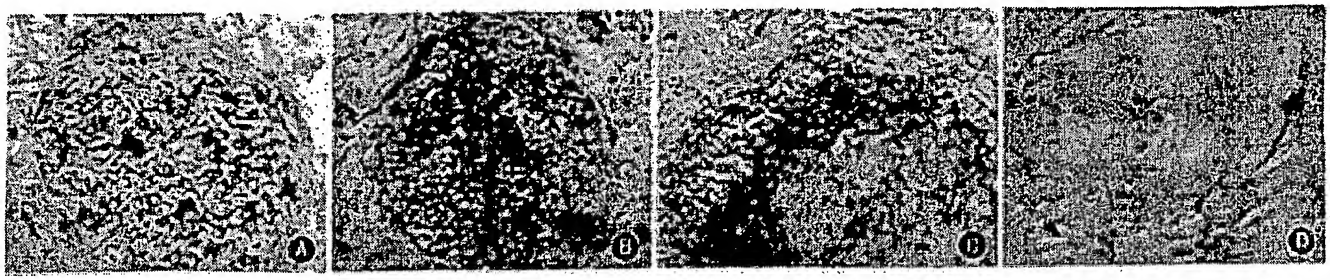

Fig. 1. Cell surface markers of vitrified human ES cells (DAB, original magnification×200). **A**: SSEA-4 positive; **B**: SSEA-3 weak positive; **C**: SSEA-1 surrounding differentiated cells are positive and undifferentiated cells in the center are negative; **D**: MEFS cells are negative for ES cell surface markers. ES: embryonic stem; MEFS: mouse embryonic fibroblasts; SSEA: stage specific expressed antigan.

(Fig. 1).

Alkaline phosphatase activity was detected (Fig. 2). RT-PCR analysis of mRNA showed that human ES cells retained the expression of OCT-4 after vitrification (Fig. 3).

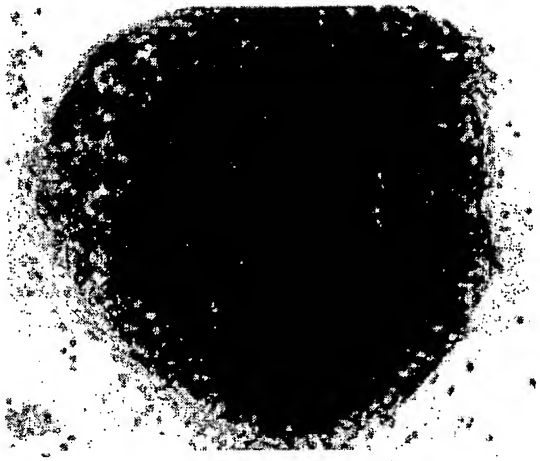

Fig. 2. Alkaline phosphatase staining of vitrified human embryonic stem cells (ALP, original magnification × 200).

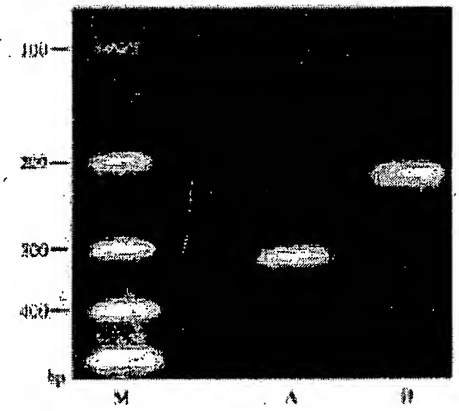

Fig. 3. The expression of OCT-4 in vitrified human embryonic stem cells. Lane M: DNA ladder; Lane A: OCT-4 (320 bp); Lane B: β-actin (200 bp).

Normal karyotypes of vitrified human ES cells (46, XX) were observed at passage 6 (Fig. 4).

Eight weeks after subcutaneous injection into SCID mice, the vitrified ES cells gave rise to multiple well-differentiated teratocarcinomas, containing a wide variety of tissues of ectoderm, endoderm, and mesoderm lineage, including ciliated epithelium, pigmented epithelium, cartilage, muscle, and bone (Fig. 5).

DISCUSSION

Conventional slow freezing and rapid thawing protocols are

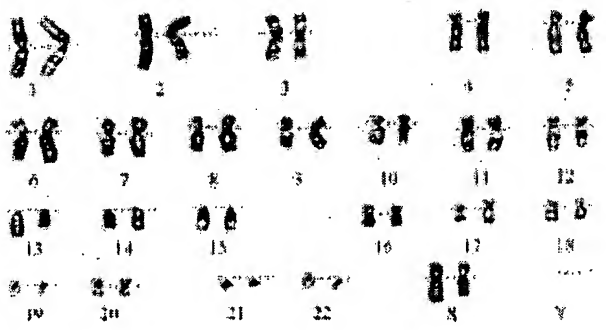

Fig. 4. Karyotype of vitrified human embryonic stem cells.

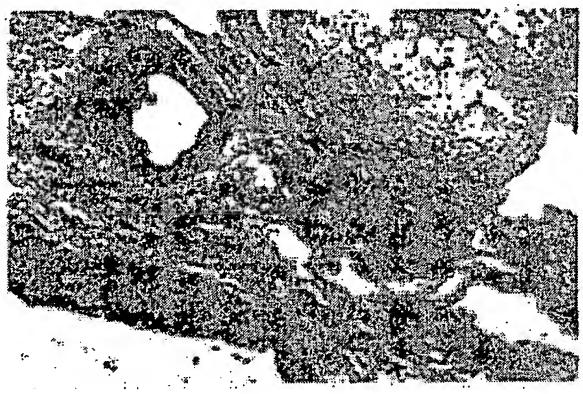

Fig. 5. Section of teratoma in host mouse derived from human embryonic stem cells (HE, original magnification ×100).

widely used in the cryopreservation of ES cell lines. Although these methods are effective for murine ES cells, which can be separated into single cells for prolonged culture, the efficiency of these conventional methods for the cryopreservation of human ES cells is relatively poor. In our study, only 22.8% of ES cell clumps could be recovered after thawing, and most of these clumps were differentiated after plating. Since human ES cells are very precious, especially in the early passages, when the total cell volume is very small and the cells differentiate more easily during prolonged culture, it is necessary to explore more effective methods for cryopreserving human ES cells.

Vitrification was first used to cryopreserve murine embryos by Rall in 1985.[6] This method has been used to preserve human oocytes and embryos.[7-9] Vitrification was recently shown to be efficient in the preservation of ova or embryos of bovine species, as well as of pigs and hamsters, in which the results of slow freezing have been variable and not always successful.[10,11] Vitrification can also be beneficial for the cryopreservation of human blastocysts.[12,13] Since human ES cells originate from the inner cell mass of the blastocyst, vitrification may also be

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efficient for the cryopreservation of human ES cells. In this study, the survival rate of vitrified ES cell clumps was 81.9%. All the recovered clumps produced colonies after plating, of which 30% remained undifferentiated, and 50% remained predominantly undifferentiated. In the slow freezing group, 16 of 70 ES cell clumps were recovered, and only one undifferentiated colony was obtained after plating. These data indicate that vitrified ES cells have a higher potential for proliferation and for remaining undifferentiated than slow freezing cells.

The main differences between vitrification and slow freezing involve the cooling rate and the cryoprotectants used. During vitrification, a glass-like solidification of the freezing solution is achieved by using a high concentration of the cryoprotectants. The cooling rate of vitrification is nearly 1500℃/min, making the intracellular and extracellular water rapidly pass through the temperature range of −5℃ to −15℃, thus avoiding the initial formation of crystals in the extracellular medium. While this approach can eliminate cell injury due to ice crystal formation, the high concentration of cryoprotectants may induce significant toxic damage. The concentrations of cryoprotectants required to achieve vitrification are inversely related to the rate of cooling. Therefore, an increased speed of cooling can lessen the cryoprotectant-induced toxicity, as it minimizes the time of exposure to these toxic compounds and allows their use at reduced concentrations. The cooling rate of slow freezing is approximately 1℃/min, which leads to extracellular crystal formation and also toxic damage. Once cell junctions are destroyed, the cells tend to detach from the clumps, and these clumps cannot be recovered after thawing.

On the other hand, the extent of the variations in osmotic pressure and the removal of DMSO from the cytoplasm during thawing are crucial for the survival of the cells. By changing the concentration of sucrose in the thawing solution, extracellular osmotic pressure affecting the vitrified ES cells decrease gradually. As a result, the cells escape from the effects of osmotic shock, and most of the intracellular low molecular weight cryoprotectants, such as DMSO, can be extracted from the cytoplasm. Thus, this stepwise dilution process helps ES cells return to normal physiological conditions. By contrast, the dilution solution in the case of slow freezing consists of the ES cell culture medium, which leads to an excessive decrease in extracellular osmotic pressure and a large influx of water into the cells, with the consequence that the cells tend to lyse and the DMSO cannot be entirely removed from the cytoplasm. This simple thawing process can cause cells to swell and broken.

A significant increase in the level of spontaneous differentiation was observed after thawing. These unwanted effects may be caused by the following three factors. First, cell injury may interfere with the normal proliferation of human ES cells. Second, during the thawing procedure, some cryoprotectants, which are strong inducing reagents of differentiation, are not removed entirely. Third, cell death during freezing and thawing may lead to the diminishing and dissociation of cell clumps, and colony growth from small clumps of cells (< 20 cells) are impossible under the conditions of routine cultures.

In our approach to vitrification, we modified the straw used for cryopreservation. Because the narrow end of a glass tube can be made thinner than that of a traditional plastic straw, the vitrification efficiency of human ES cells can be elevated by using these glass tubes. However, we still encountered some limitations. For example, only 10 − 15 cell clumps could be loaded into one straw. This may add a burden to the routine passage and cryopreservation of ES cells.

In conclusion, our study suggests that vitrification is an effective cryopreservation method for human ES cells. After thawing, undifferentiated colonies retain their pluripotency and normal karyotypes during prolonged culturing. This highly effective method would be beneficial for the cryopreservation of human ES cells at an early passage level and for the establishment of a bank of human ES cells. It would also be beneficial for the shipping of ES cells from one laboratory to another and would accelerate the study of human ES cells.

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A germ cell origin of embryonic stem cells?

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Summary

Because embryonic stem (ES) cells are generally derived by the culture of inner cell mass (ICM) cells, they are often assumed to be the equivalent of ICM cells. However, various evidence indicates that ICM cells transition to a different cell type during ES-cell derivation. Historically, ES cells have been believed to most closely resemble pluripotent primitive ectoderm cells derived directly from the ICM. However, differences between ES cells and primitive ectoderm cells have caused developmental biologists to question whether ES cells really have an in vivo equivalent, or whether their properties merely reflect their tissue culture environment. Here, we review recent evidence that the closest in vivo equivalent of an ES cell is an early germ cell.

Introduction

Embryonic stem (ES) cells are pluripotent (see Box 1) and can be expanded without limit in vitro (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). It is remarkable that permanent pluripotent stem cell lines can be derived from pre-implantation embryos at all, because, in vivo, pluripotent cells of the early mammalian embryo proliferate only briefly before becoming cells with a more restricted developmental potential. A few years after the initial derivation of mouse ES cells, it was suggested that they be called 'embryo-derived stem cells', a more precise term that would distinguish between these new pluripotent cell lines and cells within the embryo (Rossant and Papaioannou, 1984). However, this term was never adopted, and the extent to which these pluripotent stem cell lines represent any specific embryonic cell type or reflect their artificial tissue culture environment is still an open issue today – two decades later. Elucidating the origin of ES cells is of importance because it may help us to identify genes that are essential for the long-term maintenance of the pluripotent state. It could also assist with the derivation of ES cells from species whose ES cells have proved difficult to isolate. It will also help us to assess how accurately ES cell differentiation reflects events that normally occur in vivo. Here, we review the origin of ES cells, and explore recent evidence that ES cells are closely related to early germ cells.

The historical origins of ES cells: embryonal carcinoma cells

Historically, work with mouse teratocarcinomas paved the way for the derivation of ES cells. These germ cell tumors contain multiple differentiated tissues and undifferentiated stem cells, called embryonal carcinoma (EC) cells (Damjanov and Solter, 1974; Dixon and Moore, 1952; Kleinsmith and Pierce, 1964). Although teratocarcinomas had been known as medical curiosities for centuries (Wheeler, 1983), it was the discovery that male mice of strain 129 had a high incidence of testicular teratocarcinomas (Stevens and Little, 1954) that made these tumors more routinely amenable to experimental analysis. Because their growth is sustained by the persistent EC cell component (Stevens and Little, 1954), teratocarcinomas can be serially transplanted between mice. Eventually, conditions were developed that allowed the culture of EC cells in vitro, establishing them as an in vitro model of mammalian development (Kahan and Ephrussi, 1970).

As pluripotent cells of the intact early embryo proliferate for only a limited period of time, it was not initially obvious whether pluripotent cell lines could be established without undergoing malignant transformation. However, the transplantation of genital ridges or of egg-cylinder-stage embryos into ectopic sites, such as under the kidney capsule of adult mice, gave rise to teratocarcinomas at a high frequency in strains that did not spontaneously produce these tumors (Solter et al., 1970; Stevens, 1970a; Stevens, 1970b). These teratocarcinomas could be serially transplanted between adult mice, depending on whether the EC cell component persisted or differentiated (Solter et al., 1981). If the EC compartment disappears, the resulting tumor develops as a benign teratoma. Indeed, the malignant phenotype of EC cells often depends on the strain of the host mouse, and not on the tumor strain. EC cells injected into mouse blastocysts can contribute to either the normal tissues of the resulting chimera (Brinster, 1974) or, in some cases, to tumors (Rossant and McBurney, 1982). Because the ectopic transplantation of normal peri-implantation embryos can give rise to pluripotent cell lines, the direct derivation of pluripotent cell lines in vitro was attempted without the teratocarcinoma step. The culture conditions that were established to support mouse EC cells, including the use of feeder cell layers, were essentially those used to isolate mouse, and eventually human, ES cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998).

One indication that these early EC cell lines may be derived from germ cells (Solter et al., 1970; Stevens, 1967; Stevens, 1970a) came from mouse genital ridge-transplantation experiments. These experiments showed that genital ridges

Box 1. Glossary

Inner cell mass (ICM)
The second lineage of the early embryo that is located inside the blastocyst. It gives rise to all embryonic tissues.

Pluripotency
Refers to the unique ability of cells within the early embryo to differentiate into all cell types.

Primitive ectoderm
The remaining ICM tissue formed during the second differentiation event of embryonic development (also known as epiblast or embryonic ectoderm)

Primitive endoderm
An epithelial layer derived from cells that are in contact with the blastocyst cavity.

Trophectoderm
During the first differentiation event in mammalian development, morula cells segregate into two cell lineages: the first, the trophectoderm, forms the outer layer of the blastocyst. It eventually becomes part of the placenta.

effectively give rise to teratocarcinomas only in a narrow time window (E12.0-12.5). It is around this time that migratory germ cells start arriving in the genital ridge. In the egg cylinder-transplantation experiments, however, the origin of the EC cells was less clear. Primitive ectoderm seemed the most likely candidate for several reasons: because the potential to form teratocarcinomas is lost at the time when primitive ectoderm disappears at E8.5 (Damjanov et al., 1971); because EC cells have phenotypic similarities to primitive ectoderm cells in vivo (Diwan and Stevens, 1976); and because EC cells, when reintroduced into blastocysts, contribute to the same tissues as primitive ectoderm (Brinster, 1974). In addition, when analyzing the earliest stages of teratocarcinoma formation in 129/Sv mouse fetal gonads, Stevens observed clusters of polarized epithelial cells surrounding a central cavity that morphologically resembled primitive ectoderm cells (Stevens, 1983). He also observed that the formation of teratocarcinomas in ovaries included parthenogenic activation of the oocyte, the formation of blastocyst-like structures and the subsequent formation of structures that resembled early egg cylinders, which eventually became disorganized. Isolated transplanted primitive ectoderm itself gives rise to teratocarcinomas (Diwan and Stevens, 1976), but because early germ cells are just appearing at this stage, a germ cell origin cannot be completely ruled out by these experiments.

Are ES cells a tissue culture artifact?

ES cells clearly exhibit some properties that are not normally shown by cells of the intact embryo. For example, although ES cells retain properties of early embryonic cells in vitro, no pluripotent cell demonstrates long-term self-renewal in vivo. Embryonic cells, once brought into tissue culture, are exposed to numerous extrinsic signals to which they never would be exposed to in vivo. ES cells certainly adapt to selective tissue culture conditions and acquire novel functions that allow them to proliferate in an undifferentiated state indefinitely, and, because of this, ES cells are in some sense tissue culture artifacts (Buehr and Smith, 2003; Rossant, 2001; Smith, 2001).

As these changes are inevitable, the issue is not whether ES cells exhibit some properties that merely reflect their tissue culture environment, but rather whether they are most closely related to a specific in vivo cell type in the embryo, or if the influence of the culture environment is so dominant that it is impossible to relate ES cells to a single, in vivo cell type. We will certainly not completely resolve this issue here, but will re-explore the relationship of ES cells to specific early embryonic cell types.

Are ES cells most closely related to primitive ectoderm?

Although ES cell lines are generally derived from the culture of the ICM, some experiments suggest that ES cells more closely resemble cells from the primitive ectoderm. For example, isolated primitive ectoderm from the mouse gives rise to ES cell lines at a higher frequency than does isolated ICM. Moreover, the culture of primitive ectoderm allows the isolation of ES cell lines from mouse strains that have been previously refractory to ES cell isolation (Brook and Gardner, 1997). Indeed, ES cell lines can be derived from single, isolated, mouse primitive ectoderm cells, which is not possible with ICM cells (Gardner and Brook, 1997). Although these experiments suggest that ES cells are more closely related to primitive ectoderm than to ICM, they do not reveal whether ES cells more closely resemble primitive ectoderm or a cell derived from it in vitro.

A maximum of three individual cultured primitive ectoderm cells per embryo have been shown to give rise to ES cell colonies (Gardner and Brook, 1997). This low frequency could have been due to some variability in the potential of primitive ectoderm cells, to some variability in the environment in which they were placed or to damage caused by the dissociation of the primitive ectoderm into individual cells. However, by tracking the expression of the octamer-binding transcription factor 4 (*Oct4*) gene, a marker of pluripotency, in intact cultured ICM/epiblast cells, it was shown that *Oct4* expression was maintained in only a small proportion of outgrowing cells (Buehr et al., 2003), which also suggests that only a minority of primitive ectoderm cells can transit to a new stable, proliferative pluripotent state, and, subsequently, be expanded as ES cells. These results could be due to a requirement for a relatively rare intrinsic or extrinsic stochastic event, or to an inherent heterogeneity of the primitive ectodermal cell population. Recent data indicate that even the earliest ICM is heterogeneous and consists of a mixture of cells that express either *Oct4* or *Gata6* (Rossant et al., 2003), and a similar later heterogeneity could account for the fact that only a minority of primitive ectoderm cells generally give rise to ES cells in culture.

Established mouse ES cell lines express some specific markers of primitive ectoderm at a very low level, if at all (Table 1), such as fibroblast growth factor 5 (*Fgf5*) (Haub and Goldfarb, 1991; Hebert et al., 1991; Rathjen et al., 1999). Culture conditions have been established that convert mouse ES cells into early primitive ectoderm-like cells that express both *Fgf5* and *Oct4* (Rathjen et al., 1999), but these cells fail to form chimeras when injected into mouse blastocysts. Taken together, these results suggest that ES cells are most closely

Table 1. Marker genes expressed in embryonic stem cell (ES), early germ (EGC) and later germ cells (LGC), in the inner cell mass (ICM) and in the primitive ectoderm (PE)*

Gene	Species	ES	EGC	LGC	ICM	PE
Pou5f1 (Pesce and Scholer, 2001)	M	+	+	+	+	+
Nanog (Chambers et al., 2003)	M	+	+	+	+	+
Dppa3 (Saitou et al., 2002)	M	+	+	+	+	+
Ifitm3 (Saitou et al., 2002)	M	+	+	+	+	+
Kit (Horie et al., 1991)	M	+	+	+	–	N/D
DAZL (Clark et al., 2004)	H	+	+	+	–	N/D
Ddx4 (Toyooka et al., 2003)	M	–	–	+	–	–
Akp2 (Chiquoine, 1954)	M	+	+	+	+	+
Zfp42 (Rogers et al., 1991)	M	+	N/D	N/D	+	–
Fgf5 (Haub and Goldfarb, 1991; Hebert et al., 1991)	M	–	N/D	N/D	–	+
Gbx1 (Chapman et al., 1997)	M	+	N/D	N/D	+	–

*Data are based on mouse (M) and human (H) studies, some are preliminary.
+ denotes expression at that developmental stage, – denotes the gene is not expressed
N/D, not done.

related to a subpopulation of primitive ectoderm cells, or to a close derivative of primitive ectoderm cells.

One of the curious species-specific differences between human and mouse ES cells is that human ES cells give rise to trophoblast cells at a high efficiency (Xu et al., 2002), but mouse ES cells do not (Beddington and Robertson, 1989). In the intact mouse embryo, the last cells capable of giving rise to trophoblast cells are early ICM cells, so the failure of mouse ES cells to differentiate into trophoblast is good evidence that they are not the equivalent of early ICM cells (Brook and Gardner, 1997). The differentiation of human ES cells to trophoblast could be explained if they are related to an earlier cell type than mouse ES cells, or if the specification of the trophoblast lineage occurs differently in human embryos. However, a third possibility is that ES cells represent a different cell type altogether. It is therefore worthwhile examining the relationship between ES cells and germ cells.

Germ cells and the primitive ectoderm

In elegant, clonal-fate mapping studies in the mouse (Lawson and Hage, 1994), germ cells were shown to arise from a founder population in the E6.0-6.5 proximal epiblast adjacent to the extra-embryonic ectoderm. These founder cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive germ cells is present at the base of the allantois (Fig. 1) (Ginsburg et al., 1990). The E6.5 distal epiblast, which would not normally contribute to germ cells, will contribute to germ cells if transplanted to a proximal location (Tam and Zhou, 1996), which demonstrates that location and inductive signals, rather than germ plasm determinants, are responsible for the specification of germ cells in mice (Extavour and Akam, 2003). This flexibility suggests that cultured primitive ectoderm cells could spontaneously give rise to early germ cells in culture.

Bone morphogenetic protein 4 (Bmp4) (Lawson et al., 1999) and Bmp8b (Ying et al., 2000) are required for the formation of the proximal posterior extra-embryonic region that gives rise to primordial germ cells (PGCs) and to cells of the allantois in the mouse. The addition of Bmp4 and Bmp8b to distal mouse epiblast cultures increases the formation of cells strongly positive for Tnap (Ying et al., 2001), a marker shared by early germ cells and ES cells. These Tnap-positive cells were interpreted as being germ cells in this study. Recently, BMP signaling has been shown to be important for the self-renewal of mouse ES cells (Ying et al., 2003), and although BMPs are involved in many differentiation decisions in the early embryo, these results do further hint at a relationship between ES cells and early germ cells.

Similarities between germ cells and ES cells

In mice, PGCs migrate and proliferate until ~25,000 are present in the genital ridge at E13.0 (Tam and Snow, 1981). Pluripotent cell lines from pre- and post-migratory (Resnick et al., 1992; Matsui et al., 1992; Shamblott et al., 1998), as well as from migratory (Durcova-Hills et al., 2001), germ cells have been isolated, and these cell lines are termed embryonic germ (EG) cells to distinguish their origin. Mouse EG cell lines are remarkably similar to mouse ES cell lines (Donovan and de Miguel, 2003). During germ cell migration and maturation, however, the somatic status of imprinted genes is progressively erased (Yamazaki et al., 2003), and EG cells isolated at various stages of migration retain some of these differences, such as the reduced methylation of many imprinted genes, including *H19* and *Snrpn* (Hajkova et al., 2002). The analysis of mouse PGCs at E10.5 suggests that methylation erasure has already begun by this time, as supported by studies of the expression of imprinted genes (Yamazaki et al., 2003). This study showed that imprinted genes, such as *H19* and *Snrpn*, exhibit imprinted (somatic) expression patterns in E9.5 PGCs, but by E10.5 have switched to a bi-allelic mode of expression (Yamazaki et al., 2003). Because the genes expressed in ES cells exhibit somatic imprinting patterns (Geijsen et al., 2004), their change in imprinting status suggests that if ES cells are derived from germ cells, this derivation must occur before E9.5.

There is a paucity of known molecular markers that distinguish early germ cells from other pluripotent cells of the early embryo. One marker, Tnap, is strongly expressed by early germ cells and by ES cells, but is weakly expressed by the epiblast and other surrounding embryonic cells (Chiquoine, 1954; Ginsburg et al., 1990). Two new markers for early germ cells, fragilis (Ifitm3 – Mouse Genome Informatics) and Dppa3 (also know as stella or PGC7), have recently been identified that allow the better separation of early germ cell precursors from their differentiated neighboring cells (Saitou et al., 2002).

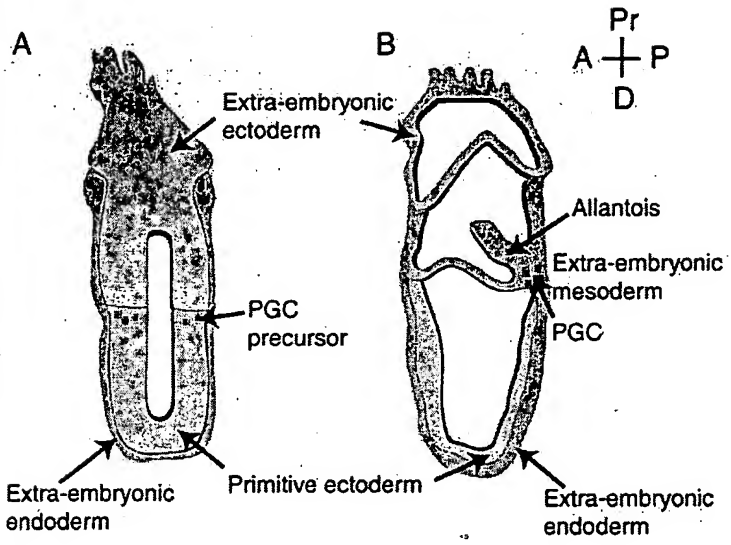

Fig. 1. Early development of the mouse embryo. (A) Six days after fertilization (E6.25), the mouse embryo consists of three layers. The inner cell mass (ICM) cells that are in contact with the blastocyst cavity differentiate into an epithelial layer called the extra-embryonic (primitive) endoderm. The rest of the ICM becomes the epiblast (primitive ectoderm). Primordial germ cells (PGCs, red dots) arise from a cell population in the proximal epiblast adjacent to the extra-embryonic ectoderm. These cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. (B) By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive PGCs is present at the base of the allantois within the extra-embryonic mesoderm (red dots). Once these PGCs are specified, they begin to migrate to the future gonadal anlagen. A, anterior; P, posterior; Pr, proximal; D, distal.

Dppa3 is expressed in pre-implantation embryos and in germ cells (Sato et al., 2002) and has recently been reported to have a role as a maternal transcript in preimplantation embryonic development (Bortvin et al., 2004). Dppa3-positive cells show increased expression of fragilis and remain positive for *Tnap* (*Akp2* – Mouse Genome Informatics) and *Oct4* (Saitou et al., 2002). Once Dppa3-positive PGCs start to migrate, they begin to express additional markers, such as steel factor receptor, followed by markers of more mature germ cells, such as murine vasa homolog (MVH; Ddx4 – Mouse Genome Informatics) (Saitou et al., 2002).

Several recent reports describing the differentiation of mouse ES cells into cells that express markers of mature male and female germ cells (Geijsen et al., 2004; Hubner et al., 2003; Toyooka et al., 2003) are important for our understanding of the origin of ES cells. In each of these reports, germ cell markers were expressed by ES cells themselves, including those, such as Dppa3, that help distinguish germ cells from primitive ectoderm (Table 1). Only the expression of more mature germ cell markers (such as MVH) enabled in vitro-derived germ cells to be distinguished from ES cells themselves. In one study that examined the differentiation of human ES cells into germ cells (Clark et al., 2004), the expression of each of eight genes that are characteristic of early germ cells was detected in human ES cells, but the expression of each of six genes that are characteristic of later germ cells was not detected, strongly suggesting that the expression of the early germ cell-genes was not merely a result of the broadly 'leaky' transcription that is often attributed to ES cells. Using immunocytochemistry, it was also shown that most individual human ES cells in a population express the early germ cell markers stella related (STELLAR) and deleted in azoospermia-like (DAZL), indicating that a minor subset of randomly differentiating cells in a mixed population is not responsible for the expression of germ cell markers in ES cell cultures. Importantly, it was also shown that at least one germ cell-specific gene, *DAZL*, was expressed by human ES cells but not by human ICM. The existing gene expression data, then, are consistent with the idea that the closest in vivo equivalent to ES cells is not the ICM or primitive ectoderm, but an early germ cell.

Some of the properties of ES cells, however, suggest that they are not merely the equivalent of early germ cells. For example, the earliest PGCs do not self-renew for prolonged periods of time, but instead begin a series of maturation steps, beginning with germ cell migration and ending in the highly specialized development of sperm or egg (Wylie, 1999). Although ES cells can differentiate into more mature germ cells in vitro, they do so relatively inefficiently. Indeed, the ability to colonize the germline of chimeras is one of the most easily lost properties of ES cells. If ES cells most closely represent early germ cells, it is unclear why they are not better at giving rise to more mature germ cells. In addition, isolated PGCs have never been demonstrated to contribute to chimeras when injected into blastocysts, so an exact equivalence to ES cells is unlikely.

Because a comprehensive and comparative analysis of the transcriptomes of isolated ICM, primitive ectoderm and early germ cells has not yet been reported, it is not yet clear how much the particular repertoire of genes expressed by ES cells represents an early germ cell, another specific in vivo cell type, a response to the tissue culture environment, or a combination of all three. If the ICM and primitive ectoderm are inherently heterogeneous, transcriptome analysis may need to be carried out at the single-cell level to ultimately understand these relationships. However, at the moment, the greatest concordance of known markers appears to be between ES cells and early germ cells.

Conclusions

We hypothesize that ES, EC and EG cells represent a family of related pluripotent cell lines, whose common properties reflect a common origin from germ cells (Fig. 2). Although a more detailed transcriptional analysis could ultimately refute the proposed relationship between ES cells and early germ cells, we hope this idea will at least help to stimulate a healthy

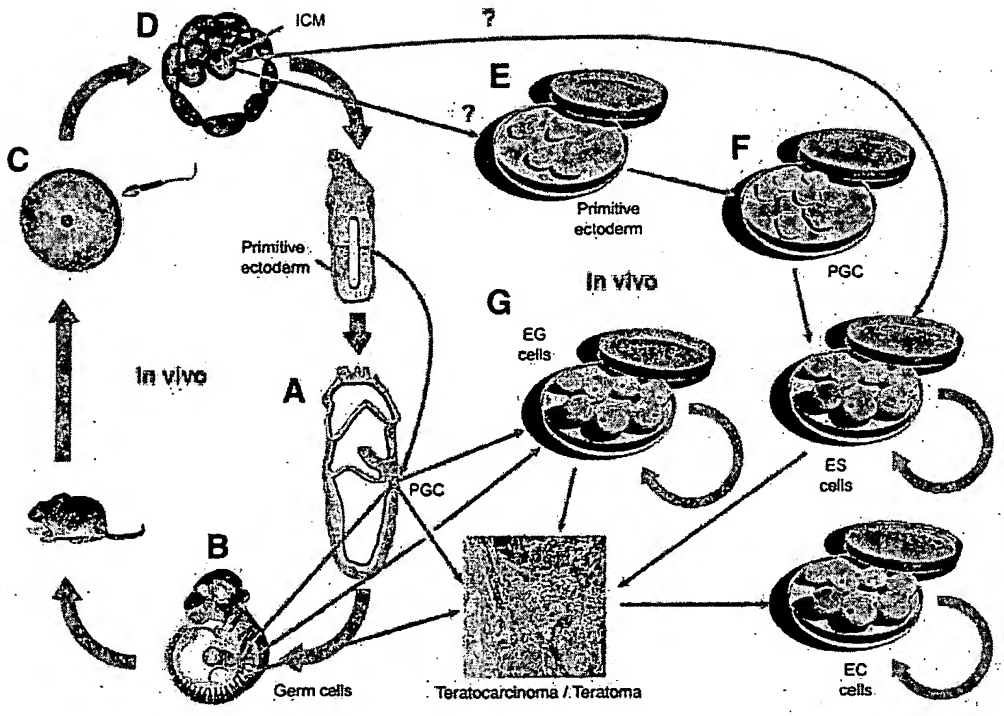

Fig. 2. Germ cell propagation in vitro and in vivo. (A-C) The germ cell cycle in the mouse. (A) Primordial germ cells (PGCs) appear at E7.25 as a small group of cells (red) in the extra-embryonic mesoderm. (B) After E8.5, PGCs start to migrate to the gonadal anlagen and contribute (C) during puberty to oocytes or sperm. (D) Embryonic stem (ES) cells are in vitro derivatives of inner cell mass (ICM) cells. (E,F) The formation of ES cells occurs either directly from the ICM/primitive ectoderm (E) or according to our hypothesis, through in vitro differentiation of ICM outgrowth into primitive ectoderm, then into extra-embryonic mesoderm and finally into PGCs (F). (G) PGCs that form in vivo (A-C) give rise to embryonic germ (EG) cells in vitro. Germ cells, PGCs, ES and EG cells are all capable of forming teratomas and teratocarcinomas. ES and EG cells can reintegrate into the normal embryo after injection into the blastocyst (not shown). Circular green arrows denote unlimited self-renewal.

re-evaluation of what is actually being studied when ES cells differentiate in vitro.

What is the relevance of a putative close relationship between ES cells and early germ cells? One prediction of this hypothesis is that at least some of the germ cell-specific genes expressed by ES cells, and not by primitive ectoderm cells, are essential for the long-term maintenance of the pluripotent state. If true, then it should be possible to generate knockout mice to identify genes that are essential for the specification or maintenance of PGCs, which are also essential for the derivation of ES cell. A related prediction of the hypothesis is that genes that are responsible for increasing susceptibility to spontaneous germ cell tumors should increase the efficiency of ES cell derivation. It is interesting, for example, that in species where teratocarcinomas occur at a clinically significant frequency, such as in mouse and human, ES cells have been successfully derived, whereas in species where teratocarcinomas are exceedingly rare, such as the rat, ES cells have proven difficult to derive. Understanding basic species differences in the specification or maintenance of early germ cells could allow the derivation of ES cells from species that have been hitherto resistant to the isolation of ES cells, such as the rat (Buehr et al., 2003).

Another implication of our hypothesis is that when looking for evolutionary clues to understand the pluripotent state, the comparative germ cell literature will be the most instructive. In a species such as the zebrafish, which has a germ plasm that strictly separates germ cells from somatic cells, it makes sense that pluripotent cell lines that can contribute to the germline in chimeras (Ma et al., 2001) would have to be derived from germ line-lineage cells.

Another prediction arising from the hypothesis that ES cells most closely represent early germ cells is that the very earliest events of ES cell differentiation into somatic and extra-embryonic lineages will not accurately reflect events that normally occur in vivo. The idea that ES cells represent an in vitro equivalent to the ICM, however, is firmly entrenched and continues to strongly influence our thinking about these cells. When examining the differentiation of ES cells in vitro, the pervasive mental image is of a forward progression that recapitulates normal embryonic events. For example, one thinks of ICM cells progressing to primitive ectoderm cells, then to neural ectoderm cells, and finally to more specialized neural cell types. If ES cells most closely represent early germ cells, this mental image needs revision, as the earliest transition would appear to be more 'lateral' or even 'backward' than 'forward'. It will be illuminating to define each of the distinct transitions that ES cells can make in a single step and to determine how much these initial transitions resemble in vivo or artificial differentiation. If ES cells really represent early germ cells, the initial events in differentiation would be expected to be transitions that do not normally occur in intact

embryos, except, perhaps, when the transition is to more mature germ cells.

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PREGNANCY POTENTIAL OF HUMAN OOCYTES — THE EFFECT OF CRYOPRESERVATION

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Abstract *Background.* In vitro fertilization, sometimes involving the cryopreservation of human embryos, has become a routine procedure for the treatment of infertility. Even though there are embryos available for transfer in about 85 percent of the treatment cycles, the rate of pregnancy rarely exceeds 25 percent per cycle. We designed this study to investigate two questions: Does this high rate of failure result from inadequate technique, or does it simply reflect the maximal potential of a cohort of aspirated eggs to produce a pregnancy? And to what extent does cryopreservation affect the capacity for implantation of embryos?

Methods. The study was conducted among patients enrolled in an egg-donation program. Aspirated eggs from a given cohort were distributed to the donor herself and a few recipients. The recipients were prepared by a standard protocol of hormone replacement and were assigned at random to the transfer of either fresh or frozen and thawed embryos. The donors received only fresh embryos.

Results. Forty cycles of donation were studied. In 25 cycles (63 percent) pregnancy was established in the donor, in the recipient (or recipients), or in both. Of the fresh embryos that were transferred to the recipients, 24 percent were successfully implanted, as compared with only 7.7 percent of the frozen and thawed embryos ($P<0.01$). A pregnancy success rate of 37 percent per recipient cycle was observed in the recipients of fresh embryos, as compared with a rate of only 16 percent in those receiving frozen and thawed embryos ($P<0.05$).

Conclusions. The majority of egg cohorts evidently possess the potential to produce a pregnancy, but cryopreservation of human embryos significantly reduces their capacity for implantation. (N Engl J Med 1990; 323: 1153-6.)

IN VITRO fertilization has become a routine procedure for the treatment of infertility, with a pregnancy success rate in many centers of 20 to 25 percent per egg collection. Considerable effort has been invested in improving laboratory procedures, methods of oocyte retrieval, and techniques of ovarian stimulation, without marked improvement in treatment results. It is not clear that a pregnancy success rate of 25 percent is the highest that can be expected. The rate depends on the potential of the retrieved eggs to produce pregnancy. In the framework of routine in vitro fertilization, it has been impossible to design and carry out an effective study of the pregnancy-producing potential of retrieved eggs, because it has been impossible to identify, isolate, and control for the numerous variables, including the different protocols used for ovarian stimulation, the quality of the sperm used, the receptivity of the uterus, and the use of cryopreservation.

An opportunity to control for some of these variables emerged from our program of egg donation, begun in 1985, in which a cohort of eggs retrieved from a donor is distributed among a group of recipients including the donor herself. Consequently, eggs from a single retrieval procedure are inseminated by different samples of semen, and the resulting embryos are transferred into different uteri. As a result, the eggs are subjected to various conditions, so that the possible effects of semen, endometrial environment, or both are controlled for. In this study, we investigated the effects of the various conditions on the rate of pregnancy, calculated per cohort of eggs. The rates we obtained may reflect the potential of a cohort to result in pregnancy.

Using the same model, we also studied the effect of cryopreservation on the implantation of embryos. The cryopreservation of human embryos is now a routine procedure in many programs of in vitro fertilization.[1-4] Its reported benefits include an increased chance of pregnancy from a single oocyte-recovery procedure[1] and a reduced risk of multiple pregnancy. Although this reduced risk would appear to be an obvious outcome of cryopreservation, it is debatable whether the chances of pregnancy per oocyte recovery are increased, especially since some embryos are likely to be damaged during cryopreservation itself. A correlation was recently reported between some morphologic features as seen on video recordings after cryopreserved

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embryos had been thawed and their capacity for implantation.[5] Very little is known about the capacity for implantation and the extent to which it may depend on the quality of the embryos or the suitability of the uterine environment. Nor is it known whether freezing and thawing affect the capacity of embryos for implantation.

In a program of egg donation, cryopreservation offers a way to match the age of the embryo with the state of the recipient's endometrium.[6] The same result can be achieved with fresh embryos, however, by applying various means of synchronization.[7,8] Our model of egg donation, in which embryos are transferred under similar conditions — either fresh or after freezing and thawing — into similarly prepared uterine environments, provided an opportunity to examine the effect of cryopreservation on the rate of implantation.

Methods

Egg Donors

In Israel the donation of oocytes is permitted only by healthy patients who have no detectable genetic diseases and who are themselves undergoing in vitro fertilization because of infertility. For our study, potential donors who fulfilled these criteria were asked to donate one third of their aspirated eggs. It was explained before they signed a consent form that such a donation might diminish their own chances of conceiving to some extent. The donors were given no information about the identity of the recipients or the fate of the donated eggs, and the identity of the donors was not known to the recipients.

Recipients

The recipients were mainly women with primary ovarian failure, with a normal uterus and an adequate endometrial response to hormone replacement therapy (as judged by endometrial biopsies). Results of the husband's sperm analysis were normal in each case. All the recipients and their husbands gave written informed consent, including a statement that the newborn would be acknowledged as their own, regardless of any complications.

Hormone Replacement Therapy

Estradiol valerate was administered, initially at a dose of 1 mg per day. The dose was increased by 1 mg every day for the next five days. The dose was then reduced to 2 mg per day, and it was maintained at that level for the remainder of the cycle. On the second day of the maintenance dose of estradiol, 100 mg of progesterone per day was added, given in vaginal suppositories. This day was considered to correspond to the day of egg retrieval in the donors.

Ovarian Stimulation, Oocyte Retrieval, and Laboratory and Freezing Techniques

Two protocols of ovarian stimulation were used in the donors. In the first, 3.2 mg of a gonadotropin-releasing hormone analogue (D-Trp-6, decapeptyl depot controlled release, Ferring, Malmö, Sweden) was given on day 21 of the natural cycle, followed two weeks later by 225 IU of human menopausal gonadotropin (Teva, Kefar Sava, Israel) per day. In the second, 225 IU of human menopausal gonadotropin a day was given starting on the third day of the natural cycle. In both protocols, human chorionic gonadotropin (Chorigon, Teva) was injected after six days of increased serum estradiol levels,[7] provided that the largest follicle reached 18 mm in diameter and estradiol levels were higher than 1835.5 pmol per liter (about 500 pg per milliliter). Oocytes were retrieved by ultrasonographically guided vaginal puncture and were cultured in modified Earle's medium. The method of freezing and thawing was that of Lassalle et al.[9] At the stage of two to four cells, the embryos were dehydrated with propylene glycol and 0.1 M sucrose and then frozen in a programmed freezer (Planer, Sunbury on Thames, U.K.). Rapid thawing was performed in the presence of 0.2 M sucrose.

Synchronization of Cycles for the Transfer of Fresh Embryos

In cycles selected for the transfer of fresh embryos, we synchronized the cycles of the recipients with that of the donor, using the so-called six-day rule,[7] which suggests that the median duration of the increase in estradiol during the follicular phase is six days. Hence, the recipients started to receive estradiol valerate on the day an increase was first noted in the donor. Exogenous progesterone was begun in the recipients on the day of egg retrieval.

Synchronization of Cycles for the Transfer of Cryopreserved Embryos

All embryos allocated for cryopreservation were frozen, regardless of their morphologic features. The recipients were treated with the same hormone-replacement protocol as those awaiting the transfer of fresh embryos. The cryopreserved embryos were thawed and transferred on the third day of progesterone therapy, which corresponded hormonally with 48 hours after egg retrieval in the donors.

Study Protocol

Thirty-two donors were treated in 40 donation cycles. The donated oocytes were distributed among 57 recipients treated in 84 cycles. Donation cycles were randomly assigned to the transfer of fresh embryos or cryopreservation. Two thirds of a donor's retrieved eggs were set aside for the donor herself, and the remaining one third was allocated to one or more recipients. The eggs thus allocated were then inseminated by the semen of the recipient's husband. Eggs originating from the same cohort were thus inseminated by different semen, and the resulting embryos, either fresh or after cryopreservation, were transferred into different uteri whose environments had all been prepared in the same way. There was no selection of embryos for freezing, and after thawing, all embryos that were not degenerating were transferred. In the donors all the available embryos were transferred fresh during the hyperstimulated cycles. In this study we defined pregnancy as any instance of established pregnancy, regardless of the number of embryos implanted. Implantation refers to the total number of implanted embryos. Embryos transferred were those that were judged suitable for replacement.

Results

Of 40 consecutive cycles of egg donation, pregnancy was established in 25 (63 percent). In 9 cycles (23 percent) either the donor or the donor plus the recipient (or recipients) conceived, and in 16 cycles (40 percent) only the recipient (or recipients) conceived. As shown in Table 1, there were no differences in the mean age of the donors, mean number of eggs aspirated per retrieval procedure, or rate of fertilization between the donor cycles that resulted in pregnancy and

Table 1. Donor Age, Number of Aspirated Eggs per Cycle, and Fertilization Rate in Donor Cycles Resulting in Pregnancy as Compared with Those Failing to Produce Pregnancy.*

Variable	Cycles Resulting in Pregnancy	Cycles with No Pregnancy	P Value
Age of donor (yr)	32.2±3.4	31±5.3	NS
No. of eggs per cycle	14±8	13±4	NS
Fertilized (%)	61	67	NS

*Plus–minus values are means ±SD. NS denotes not significant.

those that did not. The donors were treated for mechanical infertility (26 cycles), unexplained infertility (9 cycles), endometriosis (3 cycles), and male infertility (3 cycles). Eggs from 18 cycles in women with mechanical infertility produced pregnancies (69 percent) in the donors, recipients, or both. Eggs from three cycles in women with unexplained infertility led to pregnancy (33 percent), and from one cycle in a woman with endometriosis (33 percent). All three cycles involving treatment for male infertility led to pregnancies in the recipients (100 percent).

With respect to cryopreservation, of the 40 cycles of egg donation, 17 were synchronized for the transfer of fresh embryos to recipients other than the donor, and 13 of these (76 percent) resulted in pregnancy in the donors or the recipients (Table 2). Embryos from the other 23 cycles were cryopreserved, and they produced pregnancies in 12 cycles in the recipients (52 percent). (Donors received only fresh embryos.) The mean age of the donors was similar in the two groups.

The results of treatment in the recipients are shown in Table 3. The mean number of oocytes and the rate of fertilization were similar in the recipient cycles synchronized for the transfer of fresh embryos and those synchronized for the transfer of cryopreserved embryos. The implantation rate of fresh embryos, however, was significantly higher than that of frozen and thawed embryos (24 vs. 7.7 percent, $P<0.01$). Moreover, the rate of pregnancy in the group receiving fresh embryos was also significantly higher (37 vs. 15 percent, $P<0.05$).

Table 2. Comparison of Donor Cycles According to Whether Embryos Were Transferred Fresh or Frozen and Thawed.*

Donor-Cycle Cohort	No. of Cycles	Age of Donor	Eggs Aspirated per Cycle	Pregnancies per Cohort
		yr	*no.*	*no. (%)*
Fresh embryo	17	31.5±3.9	12.4±5.2	13 (76)
Cryopreserved embryo	23	32.0±3.9	15.0±7.4	12 (52)
Total	40	32.0±4.2	13.9±6.7	25 (63)

*Pregnancies in donors are included as well as those in recipients (the donors received only fresh embryos). Plus–minus values are means ±SD.

Discussion

The relatively low rate of implantation (9 to 11 percent) in most in vitro fertilization programs may be attributable to several factors associated with the procedure — the eggs, the sperm, the endometrium, and the laboratory techniques. Probably all these factors contribute in some measure to the unsatisfactory success rate of in vitro fertilization. In order to improve the rate of pregnancy, more information is needed about the contribution of each variable to the theoretical potential for pregnancy, but in the framework of a routine in vitro fertilization program it is not possible to separate the relative contributions. The use of cryopreservation permits some flexibility in the time between in vitro fertilization and the transfer of embryos, but it introduces new hazards to the embryo. Moreover, technical and ethical problems make it difficult to evaluate the results of cryopreservation of human embryos for the following reasons: embryos are in most cases preselected for cryopreservation, and normally only those with good morphologic features are considered; embryos damaged during cryopreservation are not transferred, so they are usually not included in the analysis of results; and there is no proper control group for studying the effect of cryopreservation on the implantation of embryos, because the embryos with the best morphologic features are chosen for fresh transfer during the stimulation cycle. In our study most of these difficulties were eliminated: eggs were randomly allocated for donation; there was no selection of embryos before freezing; endometria were prepared with identical protocols; and all frozen embryos were thawed and transferred regardless of morphologic features. The significantly lower rate of implantation in frozen and thawed embryos (7.7 percent) as compared with fresh embryos (24 percent) can thus be attributed to the use of cryopreservation.

Our experimental design made it possible to isolate the potential contributions of poor-quality sperm and nonreceptive endometria to the failure of implantation, because more than one sample of semen was used for each cohort of eggs, and the resulting embryos were transferred into more than one uterus. In this way we could come closer to determining the actual potential of a cohort of aspirated eggs to produce pregnancy. Our findings indicate that most (63 percent) of the cohorts aspirated in this study could result in pregnancy when provided with favorable conditions (sperm of good quality and a properly stimulated endometrium). It should be noted that the embryos from more than half the cohorts in this series were cryopreserved, resulting in a lower rate of pregnancy (52 percent). The true potential of these egg cohorts was thus

Table 3. Treatment Results in Recipient Cycles Synchronized for the Transfer of Fresh Embryos, as Compared with Those Synchronized for the Transfer of Frozen and Thawed Embryos.*

Recipient-Cycle Cohort	No. of Cycles	Eggs Inseminated	Fertilization	Embryos Transferred	Embryos Implanted	Pregnancies per Cohort
		no.	%	*no.*	*no. (%)*	
Fresh embryo	38	2.5±0.7	76	71	17 (24)†	14 (37)‡
Cryopreserved embryo	46	2.6±0.9	77	91	7 (7.7)	7 (15)
Donors	40	7.6±4.6	48	144	9 (6.25)	9 (23)

*Donors received fresh embryos only. Plus–minus values are means ±SD.

†$P<0.01$ for the comparison with the cryopreserved cohort.

‡$P<0.05$ for the comparison with the cryopreserved cohort. In three of the recipients, two embryos were implanted.

probably higher than the rate of 63 percent we obtained. When only fresh embryos were transferred, 76 percent of the egg cohorts produced a pregnancy. This pregnancy rate of 76 percent is in accord with our findings in a previous series of egg-donation cycles, in which only fresh embryos were transferred and 84 percent of the cohorts produced a pregnancy.[8] The high rate of pregnancy per cohort may be partly explained by the nature of egg donation itself: embryos that are foreign to the mother may be more effective in evoking an immune response required for implantation. Our finding that fresh embryos were implanted better in the recipients than in the donors may be also due to an immune phenomenon. A possible contribution of unrecognized male factors cannot be excluded, however, since simple semen analysis does not fully correlate with male fertility. It is also possible that an excess of embryos in the donors may actually decrease the chances of pregnancy, since multiple oocytes are most often the product of a cycle with high levels of estradiol and progesterone. The resulting overstimulation of the endometrium could be detrimental to implantation.

The findings of our study indicate that, at least in cases of egg donation, a better rate of pregnancy can be achieved using protocols of synchronization based on the transfer of fresh embryos. In contrast to this program, in which no more than three embryos were available for transfer to a recipient, a routine program of in vitro fertilization and embryo transfer might make more embryos available for transfer. The possible benefits of transferring fresh embryos could thus be complicated by the risks of multiple implantations, currently the main cause of perinatal morbidity in pregnancies involving in vitro fertilization. Acosta et al.[10] studied the effect of the number of embryos transferred on the chances of multiple implantation. They reported that in 81 patients with multiple pregnancy resulting from in vitro fertilization, 2.2 embryos per cycle were able to establish a normal pregnancy, regardless of the number of "preembryos" transferred. These findings indicate that the transfer of five embryos rather than three or four does not necessarily increase the chance of multiple pregnancy. On the other hand, two thirds of the frozen and thawed embryos in our study lost their potential to establish a pregnancy. When the number of embryos to be used in fresh transfer is being determined, both points should be kept in mind. In the absence of more reliable ways to predict the potential of a given embryo for pregnancy, the morphologic features of the blastomeres could be taken as a possible guideline. Unless there are at least two or three morphologically sound embryos available for fresh transfer, we recommend that no embryos be allocated to cryopreservation.

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Ultra-rapid freezing of mouse oocytes lowers the cell number in the inner cell mass of 5 day old in-vitro cultured blastocysts*

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We demonstrated previously that ultra-rapid freezing of mouse oocytes with 3.5 M dimethylsulphoxide (DMSO) decreased cell numbers in day 5 in-vitro cultured blastocysts. In the present study we counted cell numbers of trophectoderm (TE) and inner cell mass (ICM) separately following differential labelling of TE with propidium iodide (red) and ICM with bisbenzimide (blue). Blastocysts were from four groups of oocytes: (i) cumulus-enclosed; (ii) hyaluronidase-treated cumulus-free; (iii) cumulus-free and exposed to 3.5 M DMSO; and (iv) cumulus-free and ultra-rapidly frozen with 3.5 M DMSO. Mean (±SD) blastocyst cell numbers were 54.7 ± 22.0, 51.1 ± 17.3, 52.3 ± 13.1 and 40.4 ± 18.4, respectively. Mean TE cell numbers were 31.7 ± 18.2, 28.9 ± 13.3, 31.2 ± 13.3 and 26.2 ± 16.5 while mean ICM cell numbers were 23.0 ± 9.4, 22.2 ± 9.4, 21.1 ± 7.3 and 14.2 ± 7.3, respectively. Blastocyst and ICM cell numbers were significantly lower in the group derived from ultra-rapidly frozen oocytes compared with all other groups. Significantly more blastocysts had ≤32 cells and in blastocysts with >64 cells a lower mean percentage of ICM was found. Ultra-rapid freezing of mouse oocytes with 3.5 M DMSO can thus lead to day 5 in-vitro cultured blastocysts with significantly decreased ICM cell numbers. The residual ICM cell number in affected blastocysts may not reach a critical mass sufficient for successful post-implantation development.

Keywords: blastocyst/inner cell mass/mouse/oocyte/ultra-rapid freezing

Introduction

Technological advances in assisted human reproduction have expanded faster than our knowledge of early embryology. From 1978 on (Steptoe and Edwards, 1978), in-vitro fertilization (IVF) in combination with intracytoplasmic sperm injection (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993a,b), embryo cryopreservation (Trounson and Mohr, 1983; Zeilmaker *et al.*, 1984) and preimplantation diagnosis (Handyside *et al.*, 1990) have become available as a battery of techniques in assisted reproduction. Fundamental questions concerning the effects of these techniques on preimplantation development can only be answered if we advance our knowledge of early embryology.

One of the most critical stages in early embryology is cell differentiation in the preimplantation embryo. At blastocyst formation two cell populations are formed: the outer layer of trophectoderm (TE) that surrounds the blastocyst cavity, and the inner cell mass (ICM) which is sequestered at one pole of the blastocyst. The TE is necessary for implantation and will form the placenta and extra-embryonic membranes. The ICM forms all three germ layers and all tissues of the embryo proper as well as providing complementary contributions to extra-embryonic membranes (Gardner, 1989).

We chose as a study model the effect of ultra-rapid freezing of mouse oocytes on the cell distribution between TE and ICM in the blastocyst. Freezing of oocytes was chosen as a study subject because survival rates, rates of IVF and blastocyst formation and pregnancy rates are still low in comparison with unfrozen controls (Bernard and Fuller, 1996; Karlsson *et al.*, 1996; Van der Elst *et al.*, 1997). We had demonstrated previously that ultra-rapid freezing with 3.5 M dimethylsulphoxide (DMSO) of mouse oocytes leads to an overall decrease in the number of Giemsa-stained nuclei in 5 day old in-vitro cultured blastocysts (Van der Elst *et al.*, 1993). The aim of the present study was to investigate whether the reduced cell number in blastocysts derived from ultra-rapidly frozen mouse oocytes was due to a decrease in cell numbers in the TE, in the ICM or in both cell populations. To visualize cell differentiation we used a differential fluorescent labelling technique for TE and ICM in the blastocyst (Handyside and Hunter, 1984).

Materials and methods

Experimental design

Each experiment was performed on a single pool of mature oocytes which were randomly assigned to different subgroups: one test group of cumulus-free oocytes which underwent ultra-rapid freezing and three control groups: (i) cumulus-enclosed untreated oocytes; (ii) hyaluronidase-treated cumulus-free oocytes; and (iii) DMSO-exposed but unfrozen cumulus-free oocytes. Each experiment was repeated four times. Experiments were considered valid only when 5 day old

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in-vitro cultured blastocysts in the control group contained the expected total number of cells (± 64) of which 30–40% represented ICM cells. One experiment had to be excluded from the series, so that data are the mean from three replicates.

Oocyte collection, ultra-rapid freezing and oocyte survival

Mature oocytes were collected from hyper-stimulated female F_1 hybrid mice (C57Bl/6J×CBAca) 14 h after human chorionic gonadotrophin (HCG) injection. Ultra-rapid freezing was initiated by dehydrating cumulus-free mouse oocytes in consecutive solutions of 0.25 and 0.5 M sucrose in HEPES-buffered Earle's medium with 20% fetal bovine serum (FBS) for 5 min each. Oocytes were then exposed to the freezing solution of 3.5 M DMSO and 0.5 M sucrose for 2–3 min in the freezing straw. All manipulations were carried out at 22°C. Thawing was performed by agitating the straw for 5–6 s in a 37°C water bath. Dilution of the cryoprotectant and re-hydration of oocytes was carried out by exposure to 0.5 and 0.25 M sucrose solutions at 22°C for 5 min each followed by transfer and rinsing in sucrose-free medium. Survival of oocytes was checked under an inverted microscope at a ×400 magnification. Surviving oocytes had a clear cytoplasm, the perivitelline space was not enlarged and there was no sign of zona damage, cytoplasm leakage or clumping of cellular organelles.

In-vitro fertilization

Oocytes from the control groups and surviving frozen–thawed oocytes underwent insemination for 4 h in Whittingham's medium with 3% (w/v) bovine serum albumin (BSA). Resulting embryos were cultured in Earle's medium with 0.5% (w/v) BSA. The IVF rate was defined as the number of 2-cell embryos present 24 h after insemination compared to the number of oocytes undergoing culture after insemination. The developmental rate was defined as the number of blastocysts present 120 h after insemination compared to the number of 2-cell embryos 24 h after insemination. Blastocysts were collected at 120 h after insemination and underwent differential staining of TE and ICM.

Differential staining of ICM and TE

The cell nuclei in TE and ICM were labelled differentially with polynucleotide-specific fluorochromes. Outer TE cells were stained red with propidium iodide (PI; Sigma P-4170, Bornem, Belgium) following selective antibody-mediated complement lysis. Blastocysts were preincubated for 30 min at 22°C with whole rabbit anti-mouse serum (Sigma M-5774) and then exposed to a 1:5 guinea-pig complement solution (Sigma S-1639) containing 10 mg PI per ml at 37°C for 5 min. ICM cells are protected from lysis since the antibodies cannot pass through the junctions between the cells of the trophectoderm. Next, blastocysts were fixed and counterstained in absolute ethanol containing 20 mg per ml of the blue fluorochrome bisbenzimide (Sigma B-1155). Blastocysts were washed in absolute ethanol overnight and mounted in glycerol on glass slides under light pressure. Under UV illumination TE cells appear pink because of the dual red and blue labelling while ICM cells appear blue. Cell counting was performed blindly by one investigator under direct fluorescent microscopy at ×250 magnification. Approximately 70% of blastocysts were recovered following staining and mounting. Unambiguous total cell counting was possible for 95% of blastocysts and unambiguous differential cell counting for 65% of these. In cases where counting was not possible, the blastocysts were not sufficiently well spread or, occasionally, were floating in the glycerol. The numbers of blue and red nuclei per blastocyst were counted three times and a mean of the counts per blastocyst was registered. For each experimental group mean and SD values were calculated on the basis of the values for the individual blastocysts.

Table I. Survival, fertilization and day 5 blastocyst formation rates of four groups of mouse oocytes exposed to different treatment protocols

Treatment group	No. of oocytes	No. surviving treatment (%)	No. of 2-cell embryos (%)[b]	No. of blastocysts (%)[c]
Untreated	168		135 (80%)[c,d]	91 (67%)[f,g]
Hyaluronidase	124		82 (66%)[e]	58 (71%)
DMSO	140	132 (94%)[a]	97 (73%)	79 (81%)[f]
Frozen	290	252 (87%)[a]	173 (69%)[d]	142 (82%)[g]

Untreated: untreated cumulus-enclosed oocytes.
Hyaluronidase: hyaluronidase-treated cumulus-free oocytes.
DMSO: dimethylsulphoxide-exposed hyaluronidase-treated cumulus-free oocytes.
Frozen: hyaluronidase-treated cumulus-free oocytes ultrarapidly frozen with DMSO.
Superscripts indicate comparisons between groups: [a]$P = 0.03$, $\chi^2 = 4.7$, df = 2; [b]$P = 0.02$, $\chi^2 = 9.5$, df = 3; [c]$P = 0.009$, $\chi^2 = 6.8$, df = 2; [d]$P = 0.01$, $\chi^2 = 6.5$, df = 2; [e]$P = 0.008$, $\chi^2 = 11.8$, df = 3; [f]$P = 0.03$, $\chi^2 = 4.9$, df = 2; [g]$P = 0.005$, $\chi^2 = 8.1$, df = 2. [χ^2 with Yates' correction for continuity was used.]

Statistics

Fertilization and developmental rates and allocation of blastocysts to different cell number categories were compared by hierarchical χ^2-tests. Mean cell numbers and mean ICM:TE ratios were compared using one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparisons test. $P \leq 0.05$ was considered significant.

Results

Oocyte survival and developmental rates

The mean survival rates of 290 ultra-rapidly frozen oocytes and of 140 DMSO-exposed oocytes were 87% (range 82–95%) and 94% (range 91–100%) respectively ($P = 0.03$) (Table I).

The fertilization rate per inseminated oocyte was 80% for 168 cumulus-enclosed untreated control oocytes and was significantly lower for 124 cumulus-free oocytes treated with hyaluronidase (66%) and for the 252 oocytes which survived ultra-rapid freezing (69%) ($P = 0.02$, $\chi^2 = 9.5$, df = 3). The fertilization rate for 132 surviving DMSO-exposed oocytes was not different from that of the untreated control group.

The blastocyst formation rate per 2-cell embryo was 67% in the untreated control group and was significantly higher for DMSO-exposed oocytes (81%) and for ultra-rapidly frozen oocytes (82%) ($P = 0.008$, $\chi^2 = 11.8$, df = 3). The blastocyst formation rate for hyaluronidase-treated oocytes was 71%.

Cell numbers in ICM and TE of day 5 in-vitro cultured blastocysts

The mean cell number ± SD in 5 day old in-vitro cultured blastocysts derived from cumulus-enclosed untreated control oocytes was 54.7 ± 22.0 with 31.7 ± 18.2 cells in the TE and 23.0 ± 9.4 in the ICM (Table II). In the group of hyaluronidase-treated oocytes the mean cell number was 51.1 ± 17.3 with 28.9 ± 13.3 cells in the TE and 22.2 ± 9.4 cells

Table II. Mean cell numbers in trophectoderm (TE) and inner cell mass (ICM) of 5 day old blastocysts derived from four groups of mouse oocytes exposed to different treatment protocols

Treatment group	No. of blastocysts	Total cell number	TE cell number	ICM cell number	%ICM
Untreated	33	54.7 ± 22.0[b]	31.7 ± 18.2	23.0 ± 9.4[f]	45 ± 17%
Hyaluronidase	30	51.1 ± 17.3[c]	28.9 ± 13.3	22.2 ± 9.4[g]	45 ± 16%
DMSO	41	52.3 ± 13.1[d]	31.2 ± 13.3	21.1 ± 7.3[h]	42 ± 16%
Frozen	46	40.4 ± 18.4[a,b,c,d]	26.2 ± 16.5	14.2 ± 7.3[e,f,g,h]	38 ± 17%

For explanation of treatment groups see Table I.
Results are expressed as mean ± SD. Superscripts indicate comparisons between groups: [a]$P = 0.002$, one-way ANOVA; [b,d]$P = 0.01$, [c]$P = 0.05$, Newman–Keuls multiple comparisons test; [e]$P < 0.0001$, one-way ANOVA; [f,g,h]$P = 0.001$, Newman–Keuls multiple comparisons test.

Table III. Cell number categories in 5 day old blastocysts derived from four groups of mouse oocytes exposed to different treatment protocols

Treatment group	No. of blastocysts	% of blastocysts with		
		≤32 cells	33–64 cells	>64 cells
Untreated	33[b]	21	49	30
Hyaluronidase	30[c]	10	73	17
DMSO	41[d]	2.5	85	12.5
Frozen	46[a,b,c,d]	48	39	13

For explanation of treatment groups see Table I.
Superscripts indicate comparisons between groups: [a]$P < 0.0001$, $\chi^2 = 36.6$, df = 6; [b]$P = 0.03$, $\chi^2 = 6.9$, df = 2; [c]$P = 0.002$, $\chi^2 = 12.1$, df = 2; [d]$P < 0.0001$, $\chi^2 = 24.5$, df = 2.

Table IV. Percentage of inner cell mass (ICM) cells in different cell number categories of 5 day old blastocysts derived from four groups of mouse oocytes exposed to different treatment protocols

Treatment group	% ICM in blastocysts with		
	≤32 cells	33–64 cells	>64 cells
Untreated	49	50	34
Hyaluronidase	70	43	40[a]
DMSO	57	44	32
Frozen	40	38	23[a]

For explanation of treatment groups see Table I.
[a]$P < 0.05$, Newman–Keuls multiple comparisons test.

in the ICM. In the group of DMSO-exposed oocytes the mean cell numbers were 52.3 ± 13.1 with 31.2 ± 13.3 in the TE and 21.1 ± 7.3 in the ICM. In the group of ultra-rapidly frozen oocytes the total mean cell number was 40.4 ± 18.4 which was significantly lower than in all other groups ($P = 0.002$, one-way ANOVA). The mean cell number in the TE was 26.2 ± 16.5 which was similar to that in other groups, whereas the mean ICM cell number was 14.2 ± 7.3 which was significantly lower than in all other groups ($P = 0.001$, one-way ANOVA). The ICM:TE ratio was not significantly different between blastocysts of different treatment groups.

Categories of blastocysts according to cell number

In the group of untreated control oocytes 21% of 5 day old in-vitro cultured blastocysts had ≤32 cells, 49% had 33–64 cells and 30% had >64 cells (Table III). In the hyaluronidase-treated group 10% of blastocysts had ≤32 cells, 73% had 33–64 cells and 17% had >64 cells. In the DMSO-exposed group 2.5% of blastocysts had ≤32 cells, 85% had 33–64 cells and 12.5% had >64 cells. In the ultra-rapidly frozen group 48% of blastocysts had ≤32 cells, 39% had 33–64 cells and 13% had >64 cells. This last distribution was significantly different from those of all other groups ($P < 0.0001$, $\chi^2 = 36.6$, df = 6).

Ratio of ICM:TE in categories of blastocysts with different cell numbers

Categories of blastocysts with increasing cell numbers were defined according to the expected cell number before the fifth, between the fifth and sixth and following the sixth cleavage division, being respectively ≤32, 33–64 and >64 cells (Table IV). In each category the lowest percentage of ICM cells was consistently recorded in the group of blastocysts derived from ultra-rapidly frozen oocytes such that in blastocysts having >64 cells a significantly lower ICM:TE ratio was noted ($P < 0.05$, Newman–Keuls multiple comparisons test).

Discussion

The present study confirms and extends our previous work (Van der Elst *et al.*, 1993), where we showed that ultra-rapid freezing of mouse oocytes with 3.5 M DMSO can lead to a significant decrease in the mean total number of cells of 5 day old in-vitro cultured blastocysts. By using a differential fluorescent labelling technique for ICM and TE we were able to show here that the decrease in the total mean number of cells in the blastocyst was due to a decrease in the mean absolute number of cells in the ICM. This may be explained partly by the fact that a high proportion of day 5 blastocysts were small (≤32 cells) and that the ICM:TE ratio tended to be lower in blastocysts completing the sixth cleavage division. It is equally important to mention that a proportion of blastocysts derived from ultra-rapidly frozen oocytes were normal with respect to total cell number and ICM:TE ratio.

The decrease in cell numbers in 5 day old blastocysts derived from ultra-rapidly frozen oocytes was a consistent finding in all three replicate experiments. The validity of our data is supported by the fact that all oocytes of the four experimental subgroups in each replicate experiment were derived randomly from a single pool of oocytes. Ultra-rapid freezing has the advantage of allowing the investigator to freeze, thaw and fertilize oocytes in the same time span as

that in which control oocytes are handled. In addition, the survival rate of ultra-rapidly frozen oocytes in this study (87%) was higher than in our previous study (78%) (Van der Elst *et al.*, 1993). The fertilization rate of ultra-rapidly frozen oocytes (69%) was significantly lower than the controls (80%), as it was in our previous study (59% as compared to 69%, respectively). The high rate of blastocyst formation (82%) from 2-cell embryos derived from ultra-rapidly frozen oocytes implied that the culture conditions were appropriate.

The mean number of cells present in 5 day old blastocysts (51–55) and the presence of 40% ICM cells are similar to previous estimates of mouse blastocyst cell numbers. Using serial sectioning, Copp (1978) reported 30–150-cell blastocysts with 32–41% ICM; Chisholm *et al.* (1985) reported early mouse blastocysts with 35 cells and 31% ICM. Using both spreading and sectioning techniques, Kiessling *et al.* (1991) found a mean of 55–58 cells with 30% ICM in 5 day old blastocysts. Handyside (1978, 1981) reported 45% of ICM in 29–37-cell early blastocysts after differential staining.

The mean number of cells in the control 5 day old blastocysts in this study (51–55) indicates that they had almost completed the sixth cleavage division (64 cells) which corresponds morphologically to the expanded blastocyst stage which was visible under the microscope. Blastocyst formation commences with cavitation, which coincides with the completion of the fifth cleavage division at the 32-cell stage (Smith and McLaren, 1977). Although the blastocysts derived from ultra-rapidly frozen oocytes had only just completed the fifth cleavage division (mean of 40 cells) they already had the appearance of fully cavitated blastocysts. It has been documented that cavitation is not necessarily dependent on the number of cells (Smith and McLaren, 1977; Chisholm *et al.*, 1985) and occurs as part of a clockwork programme.

Decreased cell numbers in blastocysts may be due to delayed cleavage and/or disturbed allocation of cells to inner and outer layers. If delayed cleavage were the only cause of decreased cell numbers, then an equivalent cleavage delay would be found in TE and ICM cells, since the ratio of ICM to TE is fairly constant throughout blastocyst development. This type of delay has been described recently by Ray *et al.* (1995), who demonstrated that female human blastocysts contain fewer cells than male human blastocysts because of a 4.5 h early cleavage delay. In our study fertilization rates were decreased in ultra-rapidly frozen oocytes as well as in hyaluronidase-treated oocytes, yet only the ultra-rapidly frozen oocytes formed smaller blastocysts with reduced cell numbers in the ICM.

Allocation of cells to inner and outer layers of the compacted embryo must occur in a spatially and temporally synchronized way (Kelly *et al.*, 1978; Lehtonen *et al.*, 1988). Thus, if a blastomere is not positioned correctly or is positioned too late during the operation of the embryonic 'clockwork programme' it may not reach its destination. The origin of outer TE and inner ICM has been traced back to the late 8-cell stage when compaction occurs (Ducibella and Anderson, 1975) and when each cell forms a stable microvillous pole on its free outer surface (Handyside, 1980). Depending on the orientation of subsequent divisions, the polarized cells typically produce either one polar and one apolar daughter or two polar daughters (Johnson and Ziomek, 1981). Polar cells remain external in the morula whilst apolar cells come to reside internally (Johnson and Maro, 1986). It is the descendants of these inner cells that will give rise to the ICM when cavitation starts, marking the transition from morula to blastocyst.

Embryos with reduced numbers of cells are still able to give rise to fetuses (Willadsen and Polge, 1981; Iwasaki *et al.*, 1990; Loskutoff *et al.*, 1993; Tao *et al.*, 1995). The limits of tolerable cell loss have yet to be fully understood. Embryos produced from 1/8 blastomeres often fail to develop an ICM (Loskutoff *et al.*, 1993). It therefore appears that the ICM must have a certain critical mass to be able to form the three germ layers of the embryo. If too little ICM is present then the embryonic endoderm cannot be formed. Although only the embryonic ectoderm gives rise to the embryo proper, the interactions between the three germ layers are necessary for full embryonic and fetal development. Major defects in the ICM may lead to the formation of a trophoblastic vesicle, or the presence of too few cells in the ICM may result in preclinical abortions or blighted ova (Edwards, 1986). Iwasaki *et al.* (1990) suggested that the reduced ICM in bovine embryos following IVF as compared to those obtained by fertilization *in vivo* may be the cause of low pregnancy rates. It has also been shown that both biopsied 3/4 mouse embryos (Somers *et al.*, 1990) and halved 2-cell mouse embryos (Papaioannou and Ebert, 1995) have a significant reduction of the ICM. These findings might also have implications for embryo freezing where blastomeres are often damaged and lower pregnancy rates than from fresh embryos are found (Hartshorne *et al.*, 1990; Van der Elst *et al.*, 1995).

Further studies are needed to investigate the developmental potential of 5 day old in-vitro cultured blastocysts derived from ultra-rapidly frozen oocytes. ICM cell death is another possible cause of decreased ICM cell numbers and can be investigated by counting the number of dead cells present in TE and ICM (Handyside and Hunter, 1986). If the problem is oocyte-related, then cryopreservation of later embryonic stages should be more able to support normal development. If fertilization delay is responsible, then ICSI could be helpful since it enables timed fertilization. However, experiments in which embryos were cultured for longer than 5 days did not restore the cell number, but led to a further decrease in absolute and relative numbers of cells in the ICM (Kiessling *et al.*, 1991). In-vitro implantation experiments may provide insight into the ability of these blastocysts to reach the gastrula and early egg-cylinder stages where interaction between the germ layers comes into operation. Pregnancy data following embryo transfer into pseudopregnant females are obviously essential to determine the effect of this freezing strategy on the functional quality of the embryos. As such the present study does not demonstrate any adverse effect upon outcome of implantation and pregnancy. However, this particular experimental model using mouse oocytes in combination with DMSO and ultra-rapid freezing should not be extrapolated to other situations.

In conclusion, the relevance of this study lies in the demonstration of the possible impact of assisted reproduction techniques upon early embryology. We have shown that ultra-

rapid freezing of the mouse oocyte with 3.5 M DMSO can lead to the formation of a proportion of 5 day old in-vitro cultured blastocysts with a significant decrease in the number of cells in the ICM. This raises the question of whether the residual ICM cell number in affected blastocysts reaches the critical mass for successful post-implantation embryo development.

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Comparison of the effects of controlled-rate cryopreservation and vitrification on 2-cell mouse embryos and their subsequent development

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Effects of two cryopreservation procedures (conventional slow controlled-rate freezing using a programmable freezer and vitrification by direct plunging into liquid nitrogen) were compared on 2-cell embryos and their subsequent development to blastocysts, fresh or cryopreserved 2-cell mouse embryos were developed into blastocysts *in vitro*. The percentage of vitrified embryos which developed into blastocysts was significantly lower than that of fresh and slow controlled-rate frozen embryos. Although blastocysts from each cryopreservation procedure appeared morphologically normal and neither number of cells in the blastocysts nor in-vitro trophoblast spreading differed significantly, there were significant differences in their functional viability. First, the glucose incorporation activity in terms of [^{3}H]2-deoxyglucose (2-DG) uptake in vitrified and thawed 2-cell embryos significantly decreased compared with fresh or slow controlled-rate frozen and thawed 2-cell embryos. Second, 2-DG uptake by blastocysts developed *in vitro* from fresh 2-cell embryos and from slow controlled-rate frozen or vitrified 2-cell embryos was 105 ± 75, 43.0 ± 28.3 and 22.0 ± 11.4 fmol/embryo/h respectively. Third, the implantation rate of blastocysts developed *in vitro* from vitrified 2-cell embryos (10.2%) was significantly lower than that from fresh 2-cell embryos (30.8%) or slow controlled-rate frozen 2-cell embryos (22.1%). Since these data suggest that cryopreservation may have ulterior consequences on the functional development of embryos and that vitrification may exert a more harmful effect than slow controlled-rate freezing, more attention should be paid to its safety before vitrification is used routinely in a clinical programme.

Key words: glucose incorporation/implantation/mouse/pre-implantation embryo/slow controlled-rate cryopreservation/ultrarapid cryopreservation

Introduction

Subsequent to the first report of successful cryopreservation of mouse embryos (Whittingham *et al.*, 1972), live offspring have been produced from frozen–thawed embryos in many other mammalian species, including humans (Trounson and Mohr, 1983). Cryopreservation has become increasingly important in assisted reproductive techniques since it offers the potential advantages of reducing the risk of multiple births, while increasing the number of embryo transfers and hence pregnancies per retrieval (Van Steirteghem *et al.*, 1992). However, cryopreservation of human embryos significantly reduces their capacity for implantation (Levran *et al.*, 1990). Successful cryopreservation depends at least in part on the mode of the freezing–thawing procedure. Slow controlled-rate cryopreservation is conventionally achieved via commercially available computer-controlled cell-freezing systems. This procedure requires expensive equipment and is time consuming. Vitrification was described as a simple method of directly transferring embryos into liquid nitrogen after a brief exposure to a cryoprotectant solution (Rall and Fahy, 1985). Although this is a very attractive alternative to conventional slow controlled-rate cryopreservation, studies should have been carried out to determine its safety before using a new procedure for human embryo preservation on a routine basis.

Vitrification involves the addition of high concentrations of cryoprotectants which at extremely low temperatures are in an amorphous state without crystallizing. The original vitrification solution consisted of permeating compounds [dimethylsulphoxide (DMSO), acetamide and propylene glycol] and a macromolecular compound (polyethylene glycol) (Rall *et al.*, 1987). Later, a new cryoprotectant solution was developed (Ishida *et al.*, 1997) containing 40% ethylene glycol, 18% Ficoll and 0.3 mol/l trehalose, modifying an earlier one (Kasai *et al.*, 1990) containing ethylene glycol, Ficoll and sucrose. Ethylene glycol, a permeating compound, has an important role in stabilizing the cellular membrane during freezing, though it also has some harmful effects on embryo development (Kasai *et al.*, 1990). Ficoll is used as a low osmotic effect macromolecule to increase the viscosity of the medium. Trehalose, providing a non-permeating solution with significant osmotic effects, is a natural cryoprotectant that can be found in yeast, fungal spores, brine shrimp cysts and some soil-dwelling nematodes (Sussman and Lingappa, 1959). It seems to prevent alteration to the cellular membrane during reduced water states but the mechanism is still not well understood (Rudolph and Crowe, 1985).

Research comparing of the effect of conventional slow controlled-rate freezing and vitrification has been focused on embryo cleavage and implantation capacity and the results were controversial. Some studies have reported no statistical difference between the two procedures in blastocyst formation and implantation capacities of mouse (Rall and Wood, 1994)

or bovine (Van Wagtendonk-De Leeuw *et al.*, 1995) embryos, while Dinnyes *et al.* (1995) reported that vitrification yielded significantly higher rates of implantation than those achieved after slow freezing using mouse embryos. However, little consideration has been devoted to functional or metabolic aspects of the embryo following cooling. We have recently reported that the slow controlled-rate freezing–thawing procedure of mouse embryos decreases not only development rate to blastocyst stage, but also decreases glucose incorporation of the developed blastocysts due to decreased expression of GLUT1, suggesting that cryopreservation may have ulterior consequences on the functional development of embryos (Uechi *et al.*, 1997). We therefore decided to compare the efficacy of slow controlled-rate freezing and vitrification by assessing developmental potential of cryopreserved–thawed 2-cell mouse embryos into blastocysts *in vitro* and their ability to incorporate glucose as well as morphological features and implantation rate in recipient mice.

Materials and methods

Embryos

Eight to 10 week old Crj; CD-1 (ICR) female mice were superovulated with 5 IU of pregnant mare's serum gonadotrophin followed 48 h later by 5 IU of human chorionic gonadotrophin (HCG). Mating with males of the same strain was verified by the presence of a vaginal plug. Two-cell embryos were obtained at 44 h after HCG administration by flushing the oviducts. The embryos were placed in 2 ml of modified Biggers–Whitten–Whittigham (mBWW) medium (Biggers *et al.*, 1971), and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 48 h to obtain blastocysts *in vitro*. Randomly selected 2-cell embryos were simultaneously slowly frozen or vitrified as described below. They were thawed a few days later and then cultured in the same manner as fresh embryos. The percentage of blastocycts developed from 2-cell embryos was calculated after a total of 92 h in culture rather than the more usual 120 h post HCG to avoid allowing less viable embryos to 'catch up' with more viable ones and thus blur the differences. In-vivo developed blastocysts were collected from the uteri as described previously (Suenaga *et al.*, 1996).

Slow controlled-rate freezing and thawing procedures

Slow controlled-rate freezing and thawing of 2-cell embryos were carried out (Fugger *et al.*, 1988). The embryos were placed in 1.5 mol/l propanediol (PROH; Sigma, St Louis, MO, USA) in mBWW containing 0.3% bovine serum albumin (BSA) for 15 min at room temperature. The embryos were transferred to the same medium with 0.1 mol/l sucrose (Sigma) for 15 min and then loaded into 0.25 ml plastic straws filled with the same medium. Freezing was carried out in a programmed freezer (Cryoembryo-HP, Hoxan, Tokyo, Japan). Straws were cooled from room temperature down to –7°C at a rate of –2°C/min. Seeding was automatically induced during 15 min at this temperature. The straws were then slowly cooled down to –30°C at –0.3°C/min and then at –50°C/min to –140°C. After holding at –140°C for 5 min, they were plunged into liquid nitrogen for storage. A few days later, embryos were thawed by removing the straws from liquid nitrogen and keeping them at room temperature for 40 s. They were then hand-held until totally thawed. Cryoprotectants were removed stepwise at room temperature by transferring embryos successively (every 5 min) into mBWW supplemented with 0.3% BSA containing 1.5 mol/l PROH + 0.1 mol/l sucrose, 1.0 mol/l PROH + 0.2 mol/l sucrose, 0.5 mol/l PROH + 0.2 mol/l sucrose, and then into 0.2 mol/l sucrose. Sucrose was finally removed by placing the embryos in mBWW.

Vitrification and thawing procedures

Vitrification and thawing of 2-cell embryos were carried out (Ishida *et al.*, 1997). The embryos were placed in 40% ethylene glycol, 18% Ficoll, 0.3 mol/l trehalose in phosphate-buffered saline (PBS) containing 0.3% BSA for 5 min at 4°C. The embryos were then loaded into 0.25 ml plastic straws filled with the same medium. Straws were plunged directly into liquid nitrogen for storage. A few days later, embryos were thawed by removing the straws from liquid nitrogen and keeping them at room temperature for 40 s. They were then hand-held until totally thawed. Cryoprotectants were removed stepwise at room temperature by transferring embryos successively (every 5 min) into mBWW supplemented with 0.3% BSA containing 0.35 mol/l trehalose and then into 0.2 mol/l trehalose. Trehalose was finally removed by placing the embryos in mBWW.

Cell number of blastocysts

Blastocysts were added to Hoechst 33258 (Bisbenzimide H33258 Fluorochrome; Wako, Osaka, Japan) and left for 15 min at room temperature (Tarkowski, 1966). Observation was carried out under ultraviolet light using fluoroscein microscopy (Model BX50; Olympus, Tokyo, Japan) and the number of nuclei in each blastocyst was counted (Tsutsumi *et al.*, 1998).

Trophoblast spreading of blastocysts

Trophoblast spreading of cultured blastocysts was quantitatively analysed as described previously (Suenaga *et al.*, 1996). Blastocysts developed *in vitro* either from fresh, frozen–thawed, or vitrified–thawed 2-cell embryos were transferred to F0-CMRL medium (Suenaga *et al.*, 1996) supplemented with fetal bovine serum at a concentration of 20% (v/v), and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 96 h. The surface areas of the trophoblast spreads were quantitatively evaluated using a digitizer tablet (Model DT1000; Watanabe Sokki, Tokyo, Japan) connected to a personal computer (Model 9801, NEC, Tokyo, Japan).

2-Deoxyglucose uptake

Measurement of [^{3}H]2-deoxyglucose (2-DG, Amersham, Little Chalfont, Bucks, UK, 17 Ci/mmol) uptake was performed as described (Morita *et al.*, 1992). Fresh, frozen–thawed, and vitrified–thawed 2-cell embryos and blastocysts developed *in vitro* were incubated in 15 µl of mBWW solution containing 25 µmol/l 2-DG instead of glucose. They were incubated for 60 min at 37°C under an atmosphere of 95% air and 5% CO_2 with 100% moisture. Each embryo was then washed five times with 100 µl of glucose-free mBWW solution. The uptake of 2-DG into each embryo was counted in a Beckman scintillation counter with 1 ml of Aquasol solution.

Embryo donation model

Seven blastocysts of each experimental group, i.e. blastocysts developed *in vitro* either from fresh, frozen–thawed, or vitrified–thawed 2-cell embryos were transferred surgically to the tip of one or the other uterine horn in the recipient mice on day 3 of pseudopregnancy as described previously (Morita *et al.*, 1994). On day 9 of gestation, the recipients were killed and autopsied and the implantation rate was calculated as the ratio of implanted embryos to transferred blastocysts.

Statistical analysis

Statistical analysis was performed using Student's *t*-test and the χ^2-test. Statistical significance was established at the $P < 0.05$ level.

Results

After slow controlled-rate freezing and vitrification, 81.6% (746/914 embryos) and 77.4% (230/297) of 2-cell embryos were recovered with normal morphology respectively. In-vitro developed blastocysts were obtained by explanting either fresh, frozen–thawed, or vitrified–thawed 2-cell embryos and allowing them to develop *in vitro* for 48 h. The percentage of normally developed blastocysts from slowly frozen–thawed 2-cell embryos (32.8%; 198/603 embryos) was significantly lower compared with that of fresh embryos (47.1%; 248/526 embryos) (Figure 1). The control blastocyst formation rate starting from 2-cell embryos (47.1%) was rather low and may have been related to the mouse strain used (see below). It may also have been caused by us making our observations at 92 h post HCG rather than at 120 h which is more usual. The lowest blastocyst formation rate (22.3%; 124 in 556 embryos) was observed when vitrified–thawed 2-cell embryos were cultured *in vitro*, which was significantly lower than those of fresh and slowly frozen–thawed embryos (Figure 1). Morphologically, those blastocysts developed *in vitro* with or without cryopreservation could not be distinguished from those developed *in vivo* obtained 92 h following HCG administration from the mouse uteri. In addition, the number of cells in these blastocysts developed from frozen– and vitrified–thawed 2-cell embryos did not differ significantly from those developed *in vitro* from fresh 2-cell embryos (Table I). On the other hand, blastocysts from fresh embryos exhibited a significantly faster rate of trophoblast spreading after 96 h culture (Figure 2A) than those from frozen–thawed (Figure 2B) and vitrified–thawed (Figure 2C) 2-cell embryos, but there was no significant difference between the two cryopreservation procedures (Table II).

Table I. The number of cells in the blastocysts developed *in vitro* from fresh, slowly frozen–thawed, and vitrified–thawed 2-cell mouse embryos

Treatment	No. of cells
None (fresh)	39.4 ± 6.6 (21)
Slowly frozen–thawed	35.8 ± 4.7 (13)
Vitrified-thawed	36.2 ± 3.4 (11)

Values are mean ± SD cells/blastocyst. Numbers of sampled blastocysts appear in parentheses. There was no significant difference in the number of cells between these three groups of blastocysts.

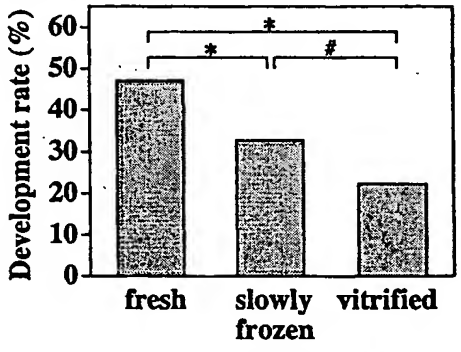

Figure 1. Development rates of fresh, slowly frozen–thawed, and vitrified–thawed 2-cell mouse embryos to blastocysts. Two-cell embryos were cultured *in vitro* for 48 h to yield blastocysts. *$P < 0.001$ compared with development rate of fresh 2-cell embryos. #$P < 0.05$ compared with that of slowly frozen–thawed 2-cell embryos.

2-Deoxyglucose (2-DG) uptake in 2-cell embryos and blastocysts developed *in vitro* from 2-cell embryos with or without cryopreservation is shown in Figure 3. Slow controlled-rate freezing–thawing procedure itself did not alter the uptake of 2-DG in 2-cell embryos. However, 2-DG uptake by 2-cell embryos after vitrification was significantly lower than that of fresh 2-cell embryos and that after slow controlled-rate cryopreservation (Figure 3). There was significant increase in the uptake of 2-DG in the blastocysts developed *in vitro* compared with the corresponding 2-cell embryos irrespective of presence or absence of cryopreservation (Figure 3). 2-DG uptake increased >6-fold in the blastocysts developed *in vitro* compared with the fresh 2-cell embryos. An ~3-fold significant increase in 2-DG uptake was observed in blastocysts developed from slowly frozen–thawed embryos. However, glucose incorporation of the blastocysts developed from slowly frozen–thawed embryos was significantly lower than that developed from fresh 2-cell embryos. Significant increase was observed

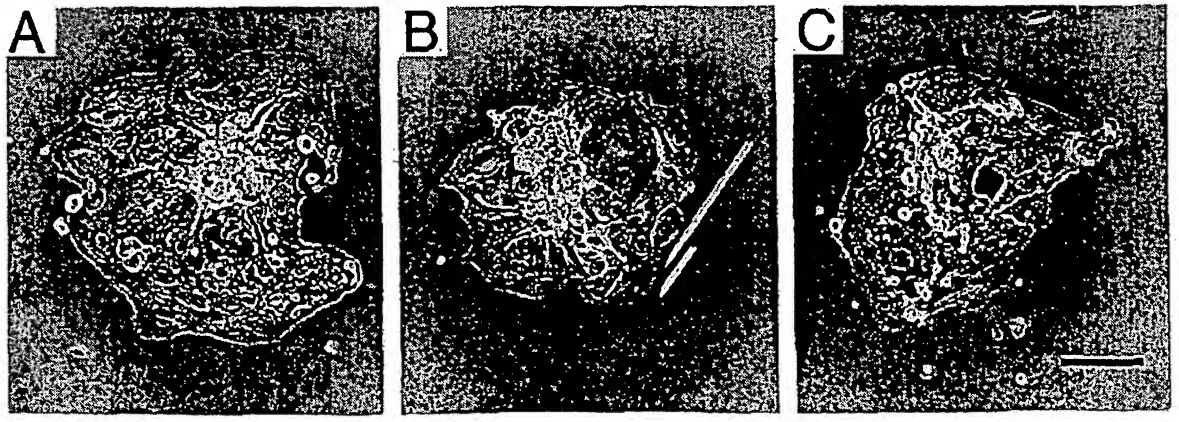

Figure 2. Representative photomicrographs showing the trophoblast spreading after 96 h culture of blastocysts developed from fresh (A), slowly frozen–thawed (B), and vitrified–thawed (C) 2-cell embryos. Bar = 100 μm.

Table II. Comparison of the surface areas of the trophoblast spreading after 96 h culture of blastocysts with or without cryopreservation procedure

Treatment	Surface area (%)
None (fresh)	100 ± 9.7[a]
Slowly frozen–thawed	78.1 ± 6.0[a]
Vitrified–thawed	82.3 ± 4.8

Values are mean ± SE of the mean trophoblast spreading of the blastocysts developed from fresh 2-cell embryos. Number of samples was 15 in each group.
[a]Significant difference: $P < 0.05$.

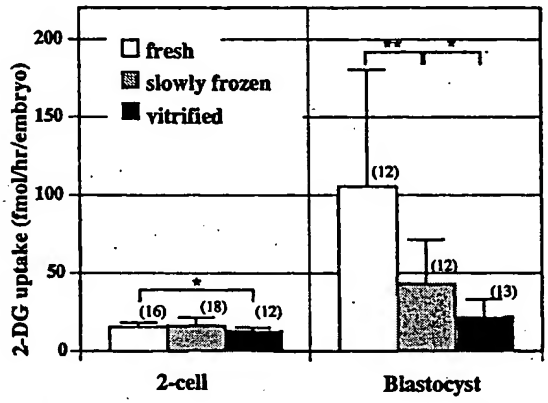

Figure 3. 2-Deoxyglucose (2-DG) uptake in mouse 2-cell embryos and blastocysts developed from fresh (open bars), slowly frozen–thawed (hatched bars), and vitrified–thawed (closed bars) 2-cell embryos. The 'T' on the top of the bars indicates SD. Numbers of samples of embryos and blastocysts appear in parentheses. There was significant increase in the uptake of 2-DG in the blastocysts developed *in vitro* compared with the corresponding 2-cell embryos, $P < 0.01$ for fresh and slowly frozen–thawed embryos, $P < 0.05$ for vitrified–thawed embryos. Significant differences: $^{*}P < 0.05$, $^{**}P < 0.001$ respectively.

in 2-DG uptake also in blastocysts developed from vitrified–thawed embryos, but the glucose incorporation of these blastocysts was significantly lower than those blastocysts that had not undergone the freezing–thawing procedure and those that experienced the slow controlled-rate freezing–thawing procedure (Figure 3).

The highest rate of implantation was observed when blastocysts developed *in vitro* from fresh 2-cell embryos were transferred to recipient mice; 41/133 embryos (30.8%) implanted successfully (Table III). The implantation rate of blastocysts developed from slowly frozen–thawed embryos was lower than that from fresh ones but no significant difference was observed between these two groups of blastocysts. The lowest implantation rate was observed when blastocysts developed from vitrified–thawed 2-cell embryos were transferred, which was significantly lower compared with that of blastocysts developed *in vitro* from fresh or slowly frozen–thawed embryos (Table III).

Table III. Implantation rates of blastocysts developed *in vitro* from 2-cell embryos with or without cryopreservation

Blastocysts developed from	Transferred (*n*)	Implanted (*n*)	Implantation rate (%)
Fresh 2-cell embryos	133	41	30.8
Slowly frozen–thawed 2-cell embryos	77	17	22.1
Vitrified–thawed 2-cell embryos	98	10	10.2[a,b]

Two-cell embryos were cultured *in vitro* for 48 h to yield blastocysts. Blastocysts were transferred to uterine horn of pseudopregnant recipient mice.
[a]$P < 0.001$ compared with implantation rate of blastocysts developed *in vitro* from fresh 2-cell embryos.
[b]$P < 0.05$ compared with that of blastocysts developed *in vitro* from slowly frozen–thawed 2-cell embryos.

Discussion

The rate of embryonic development differs between cryopreserved embryos and fresh embryos as shown here (Figure 1) and previously (Fugger *et al.*, 1988; Selick *et al.*, 1995; Uechi *et al.*, 1997). In the present study, the percentage of development to blastocysts from vitrified–thawed 2-cell embryos, after 48 h culture, was significantly lower compared with that from fresh and slowly frozen–thawed 2-cell embryos (Figure 1). These data confirm that cryopreservation of early embryos is lethal to some embryos and suggest that vitrification, though simple and less time consuming, is more detrimental than conventional slow controlled-rate cryopreservation, when performed on mouse 2-cell embryos.

In contrast with our present study, there have been many studies so far reporting good results with vitrification. Two main reasons may explain our obviously poor rates of blastocyst development, i.e. mouse strain and the developmental stage at which mouse embryos undergo cryopreservation. The Crj; CD-1 (ICR) mouse strain we used is inbred, showing a developmental rate of ~50% as shown in our present study and recent publication (Tsutsumi *et al.*, 1998). Our vitrification protocol is almost the same as that previously reported (Ishida *et al.*, 1997), but they used a B6C3F1 hybrid mouse strain. Ethylene glycol-based cryoprotectant solution similar to that in our present study had previously been used (Kasai *et al.*, 1990) but with morula stage embryos. Using a later developmental stage such as 8-cell and morula caused a higher proportion of vitrified embryos to develop to blastocysts (Miyake *et al.*, 1993). 8-cell embryos of an ICR mouse strain were used (Mukaida *et al.*, 1998) and they reported that ethylene glycol-based cryoprotectant solution was more suitable for vitrification than PROH-, DMSO-, acetamide-, or glycerol-based solution. The reason that we used 2-cell embryos of ICR mouse strain is that we assumed that any difference in detrimental effect between the two cryopreservation procedures may be emphasized if 'suboptimal' developmental stage embryos of a 'suboptimal' strain is used instead of optimal stage embryos of an optimal strain, as previously shown (Kasai *et al.*, 1990; Ishida *et al.*, 1997). Moreover, we believe that our data obtained using 2-cell embryos of ICR mice are helpful for the improvement of the freezing–thawing procedure for human embryos in clinical practice which sometimes do not have optimal quality.

These data concerning the rate of embryonic development to blastocysts also raise a question whether or not slow controlled-rate freezing and vitrification procedures exert an

'all or nothing' effect on these mouse embryos. Therefore, we focused on the comparative effects of these cryopreservation procedures on the quality and viability of those embryos that survived and developed into blastocysts. However, blastocysts developed *in vitro* from slowly frozen–thawed or vitrified–thawed 2-cell embryos could not be morphologically distinguished from blastocysts developed from fresh 2-cell embryos and the number of cells in the blastocysts did not differ significantly between the three groups (Table I). It has been reported that ultrarapid freezing of mouse oocytes lowers the cell number in the inner cell mass of day 5 blastocysts (96 h after 2-cell embryos) (Van der Elst *et al.*, 1998). Similarly, in the present study, there was a slight decrease in the surface area of trophoblast spreading after 96 h culture (120 h after 2-cell embryos) of blastocysts developed from cryopreserved 2-cell embryos compared with fresh 2-cell embryos. However, there was no significant difference between the two cryopreservation procedures (Figure 2 and Table II). Thus, we attempted to assess the viability of embryos by measuring their glucose uptake because current experimental data indicate that there is an alteration in the uptake or metabolism of glucose in early stage embryos (Leese and Barton, 1984; Khurana and Wales, 1987; Butler *et al.*, 1988; Brison and Leese, 1991; Morita *et al.*, 1992). Indeed, it has been shown that glucose uptake can be used to select prospectively viable blastocysts immediately after thawing (Gardner *et al.*, 1996).

In the present study, it is of interest to note that 2-DG uptake in 2-cell embryos was significantly decreased by vitrification compared with those of fresh or slowly frozen–thawed 2-cell embryos (Figure 3). It is postulated that vitrification itself causes an alteration in embryonic quality by affecting functional integrity of the 2-cell embryos. Cellular glucose uptake is dependent upon a family of glucose transporter proteins that contain multiple membrane-spanning domains (Birnbaum *et al.*, 1986; James *et al.*, 1989; Orci *et al.*, 1989). A more plausible explanation for this decrease in glucose incorporation activity is that during the vitrification–thawing procedure, glucose transporters in the membrane of the blastomeres of embryos are damaged, resulting in a decreased 2-DG uptake in the embryos.

The 2-DG uptake in blastocysts was significantly higher than the respective 2-cell embryos in all three groups. However, 2-DG uptake in the blastocysts developed *in vitro* from cryopreserved–thawed 2-cell embryos was significantly lower than that of the blastocysts developed *in vitro* from fresh 2-cell embryos (Figure 3). As we have already reported (Uechi *et al.*, 1997), this decrease in 2-DG uptake of morphologically normal embryos may reflect a delayed effect of cryopreservation, suggesting that it may have ulterior consequences on the functional development of embryos. Moreover, 2-DG uptake of the blastocysts developed from vitrified–thawed 2-cell embryos was significantly lower than that of the blastocysts developed from slowly frozen–thawed 2-cell embryos. These data indicate that different types of freezing–thawing procedure have different degrees of delayed effect as detected by 2-DG uptake, and that it is not an 'all-or-nothing' type of effect that is assessed by 2-DG uptake assay. Since glucose incorporation activity is dependent on glucose transporter GLUT1 expression in early embryos (Morita *et al.*, 1994) and impaired GLUT1 expression is reported in slowly frozen–thawed embryos (Uechi *et al.*, 1997), further investigation into mechanisms responsible for the gene expression may help to understand the impact of cryopreservation on the metabolic activity of embryos.

The viability of each group of embryos was assessed also by an embryo donation model that provides a way of determining whether a developmental failure occurs due to a defect in the embryo or in the environment. The implantation rate of blastocysts developed from vitrified–thawed 2-cell embryos was significantly diminished compared with that of blastocysts developed from fresh and slowly frozen–thawed 2-cell embryos (Table III). This suggests that the quality of the blastocysts developed after vitrification might be impaired as a result of delayed effects or consequences and thus implantation capacity might be reduced although they were morphologically indistinguishable from those obtained after slow controlled-rate freezing in terms of cell number and trophoblast spreading. This has implications for the application of cryopreservation technology as well as for cryobiology. Empirical studies are necessary for continued development of techniques that will maximize success rates and minimize time and expense of cryopreservation procedures and thus mechanisms of cryoinjury and its prevention may be understood.

Studies on perinatal outcome and follow-up of babies conceived from cryopreserved embryos have shown no pathological features (Wada *et al.*, 1994; Olivennes *et al.*, 1996). However, embryos that survive to blastocysts following vitrification may have some cryoinjury not in an 'all or nothing' way since they appear to be normal in morphology and are capable of further development. It was reported recently that slow controlled-rate freezing is more efficient than ultrarapid cooling, not vitrification, for human embryos (Van den Abbeel *et al.*, 1997). It remains to be determined whether offspring from vitrified embryos are phenotypically and genetically normal in all regards. Before vitrification is used routinely in clinical in-vitro fertilization programmes, its safety must be convincingly demonstrated.

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Nutrient Uptake and Utilization Can Be Used to Select Viable Day 7 Bovine Blastocysts After Cryopreservation

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ABSTRACT The ability of individual bovine blastocysts to survive freezing and thawing procedures was assessed by measuring glucose and pyruvate uptake and lactate production immediately before and after cryopreservation. Using glucose and pyruvate uptake and lactate production it was not possible to determine, prior to freezing, which blastocysts would be viable after thawing. However, in the 5 hr immediately after thawing, those blastocysts which expanded their blastocoel had significantly greater glucose and pyruvate uptake and lactate production ($P < 0.01$) than those embryos which failed to develop after a 14 hr overnight incubation. Interestingly, after thawing, two distinct populations of blastocysts existed with respect to glucose uptake and lactate production, indicating that it is possible to identify those blastocysts immediately after thawing which will reexpand. In contrast, there was a considerable degree of overlap in pyruvate uptakes between the viable and nonviable groups of embryos, indicating that this parameter could not be used to select viable embryos after thawing. There was an increase in the calculated oxidation of carbohydrates after thawing, consistent with a partial uncoupling of the inner mitochondrial membrane. In conclusion, glucose uptake and lactate production can be used to select prospectively viable blastocysts immediately after thawing, indicating that glycolysis is a major energy-generating pathway for the embryo at this time.

Key Words: Viability, Glucose, Lactate, Glycolysis, Freezing

INTRODUCTION

Assessment of embryo viability in culture remains relatively subjective, and morphology is the principal criterion used to select blastocysts for transfer to recipients (Gardner and Leese, 1993). Development of suitable quantitative methods for determining embryo viability would facilitate the selection of embryos for transfer or cryopreservation, thereby increasing the success rates of assisted reproductive technologies. Nutrient uptake has previously been correlated with developmental capacity after transfer at the blastocyst stage in both cattle and mice. In retrospective analyses, glucose uptake by day 10 bovine blastocysts (Renard et al., 1980) and day 4 mouse blastocysts (Gardner and Leese, 1987) in vitro was positively correlated with viability after transfer to recipient females. More recently, metabolic activity has been used to select prospectively viable mouse blastocysts prior to transfer. Using glucose conversion to lactate to quantitate glycolysis, it was possible to identify embryos from a population of morphologically identical mouse blastocysts which would develop after transfer and those embryos which had little, if any, developmental potential (Lane and Gardner, 1995). In the present study we therefore used both nutrient uptake and utilization to assess the ability of day 7 bovine blastocysts to continue to develop in vitro after cryopreservation.

MATERIALS AND METHODS

Generation of Blastocysts

Blastocysts were generated by the in vitro maturation and fertilization of ovarian oocytes. Follicles of 2–5 mm diameter were aspirated with a 19-gauge needle using vacuum suction. Oocytes with at least five distinct layers of cumulus cells were selected and matured in groups of 50 for 24 hr in 800 μl of maturation medium (TCM-199 supplemented with 25 mM sodium bicarbonate, 10% fetal calf serum, 0.01 U/ml of LH and FSH). Oocytes were matured for 24 hr at 39°C in an atmosphere of 5% CO_2 in air. At the completion of culture for maturation, oocyte cumulus complexes were washed twice in TCM-199 buffered with HEPES, once in the fertilization medium (Fert-TALP; Bavister, 1989), and then placed in 30 μl of this medium ready for insemination. Sperm were prepared by centrifugation (600*g* for 20 min) over a 45:90% Percoll density gradient. Excess Percoll was removed by washing in 5 ml of Fert-TALP medium prior to insemination. Ten oocytes were then inseminated with 2×10^6 sperm/ml in a total volume of 50 μl Fert-

Abbreviations: LH, Luteinising Hormone; FSH, Follicle Stimulating Hormone; HEPES, Hydroxyethylpiperazine ethanesulfonic acid; SOF, Synthetic Oviduct Fluid; BSA, Bovine Serum Albumin; NAD(P)H, Nicotinamide Adenine Dinucleotide (Phosphate); PBS, Phosphate Buffered Saline; ATP, Adenosine Triphosphate.

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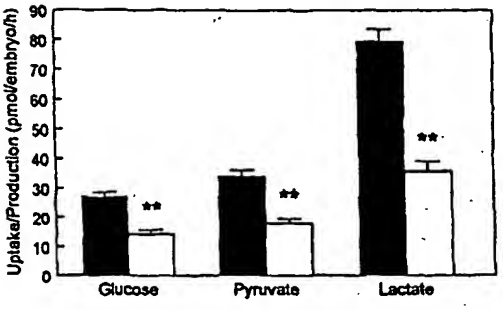

Fig. 1. Glucose and pyruvate uptake and lactate production by individual day 7 bovine blastocyst before (solid bars) and after (open bars) freezing. Values are mean ± SEM. **Significantly different from before freezing; $P < 0.01$.

TALP medium. Gametes were incubated for 22 hr at 39°C in an atmosphere of 5% CO_2 in air. At the completion of culture for insemination, the putative zygotes were denuded of surrounding corona and cumulus cells by vortexing for 2 min. Pronuclear oocytes were subsequently washed three times and then cultured for 6 days in SOF medium supplemented with amino acids (SOFaa) and BSA (8 mg/ml) in 5% CO_2/7% O_2/88% N_2 at 39°C (Gardner, 1994; Gardner et al., 1994). Embryos were cultured in groups of four in 30-μl drops of medium under mineral oil. The medium was renewed every 48 hr. Using this standard procedure, approximately 40% of oocytes matured (45% of fertilized oocytes), and reached the expanded blastocyst stage after 6 days of culture (day 7 of development) (Gardner, 1994; Trounson et al., 1994).

Medium TCM199, sodium pyruvate, sodium lactate, amino acids, and phenol red were obtained from Sigma Chemical Co. (St. Louis, MO). All salts and glucose were of Analar grade (BDH, Poole, Dorset, United Kingdom). Antibiotics were purchased from CSL (Parkville, Victoria, Australia), and HEPES from Calbiochem (Alexandra, New South Wales, Australia). All SOF media were supplemented with lipid-stripped BSA (BSA-ET) from Life Technologies (Mulgrave, Victoria, Australia).

Assessment of Embryo Metabolism and Cryopreservation Procedure

Blastocysts with an expanded blastocoel and distinct inner cell mass and equivalent diameter were selected for metabolic assessment, washed three times, and subsequently incubated in 1 μl of modified SOFaa (with 0.5 mM glucose, 0.5 mM pyruvate, and no lactate) for 5 hr in 5% CO_2/7% O_2/88% N_2 at 39°C. A sample of medium was taken for metabolite analysis at completion of incubation, and concentrations of glucose, pyruvate, and lactate were determined by microfluorimetry (Gardner and Leese, 1993; Gardner et al., 1993). The fluorometric assays were miniaturizations of conventional metabolite assays, employing the pyridine nucleotides NADH. Assay reactions were housed in nanoliter drops on microscope slides under mineral oil. Fluorescence of reactions was measured using a fluorescence microscope with photomultiplier and photometer attachments, and a series of standards run each day to calibrate fluorescence.

Blastocysts were cryopreserved by slow freezing. Embryos were initially placed in 5% glycerol (in PBS) and 0.05 M sucrose for 5 min, and then transferred to 10% glycerol (in PBS) and 0.1 M sucrose for a further 20 min prior to being loaded individually into freezing straws. Blastocysts were cooled at 0.3°C/min to −35°C, at which temperature the freezing straws were plunged into liquid nitrogen. The following morning, blastocysts were removed from the liquid nitrogen and thawed in air for 10 sec and then placed in a 37°C water bath for 15 sec. Individual blastocysts were then incubated in 10% glycerol in PBS with 0.1 M sucrose for 1 min, after which they were transferred to 5% glycerol (in PBS) with 0.05 M sucrose for 6 min. Finally, blastocysts were placed in 0.05 M sucrose for 6 min before being incubated in HEPES-buffered SOF for 10 min. Blastocysts were subsequently washed three times in modified medium SOFaa and then incubated in a further 1-μl drop of modified SOFaa for 5 hr. A further sample of medium was taken for metabolite analysis at the completion of this incubation, and the embryos were transferred individually to a 30-μl drop of SOFaa and cultured for 14 hr. Blastocyst reexpansion (viability) was recorded, and those blastocysts which failed to reexpand were classified as nonviable.

Statistical Analysis

Differences between groups were determined by t-test.

RESULTS

Both nutrient uptake and utilization by blastocysts were significantly reduced after freezing ($P < 0.01$; Fig. 1). When embryos were subsequently sorted into

TABLE 1. Effect of Freezing on Bovine Blastocyst Metabolism Before and After Freezing†

	Before (viable)	After (viable)	Before (nonviable)	After (nonviable)
Glucose uptake (pmol/embryo/hr)	28.4 ± 2.2	22.7 ± 1.6*a	25.4 ± 2.3	4.0 ± 0.7***a
Pyruvate uptake (pmol/embryo/hr)	35.2 ± 2.9	24.6 ± 1.7**b	32.6 ± 3.0	9.9 ± 2.2***b
Lactate production (pmol/embryo/hr)	84.4 ± 5.7	54.7 ± 3.2***c	73.4 ± 5.6	13.0 ± 2.4***c

†Values are mean ± SEM. Viable, n = 38; nonviable, n = 32.
*Significantly different from before freezing; $P < 0.05$.
**Significantly different from before freezing; $P < 0.01$.
***Significantly different from before freezing; $P < 0.001$.
a–cValues with the same letter are significantly different; $P < 0.001$.

reexpanded (viable; n = 38) and degenerate (nonviable; n = 32) groups after thawing, it was evident that the observed decline in nutrient uptake and utilization could be largely attributed to those embryos which failed to continue development after freezing and thawing (Table 1). However, it was not possible to identify which blastocysts would survive the freezing procedure, as both viable and nonviable embryos had similar nutrient uptakes and lactate production prior to cryopreservation.

The range of glucose uptakes after thawing by viable and nonviable embryos did not overlap, and separated into two distinct populations (Fig. 2). Similarly, there was little overlap in lactate production between the two populations.

Prior to freezing, only 9% of the carbohydrate taken up by the viable embryos was assumed to be oxidized (only 8 pmol of the carbohydrate taken up could not be accounted for by lactate production, assuming that 1 mol of glucose can result in 2 mol of lactate, and that 1 mol pyruvate can result in 1 mol lactate). However, after thawing, this figure was increased to 23%. Similarly, in the nonviable group of embryos, the amount of carbohydrate that was theoretically oxidized increased from 12% before freezing to 28% after freezing.

DISCUSSION

It was not possible, using nutrient uptake and utilization, to determine prior to freezing which blastocysts would survive the procedure. In contrast, both glucose uptake, and to a lesser degree lactate production, could be used to identify, within the first 5 hr after thawing, which blastocysts would continue to develop in vitro before it was possible to determine which embryos would expand using morphological criteria. Even in the viable group of embryos there was a significant reduction in all metabolic parameters measured after freezing, indicating that the blastocyst's metabolic rate was not immediately restored to prefreezing levels. It appears that substrate oxidation is increased after freezing, which is consistent with an increased energy demand associated with reexpansion. However, this could not be associated with viability, as both groups of embryos exhibited a similar decline in percentage of lactate production. Oxygen uptake by bovine blastocysts has been proposed as a marker of embryo viability (Overstrom et al., 1992). A more plausible explanation for the calculated increase in oxygen consumption is that during the cryopreservation procedure, the inner membranes of the mitochondria are damaged, resulting in a partial uncoupling of oxidative phosphorylation, leading to increased oxygen consumption without a corresponding increase in ATP production (Tsvetkov et al., 1986). As a result of this, those blastocysts which can compensate by an increase in glycolytic activity would be better able to survive and develop after freezing and thawing. Indeed, Rieger et al. (1993) showed that after thawing, bovine blastocysts which subsequently hatched had a greater glycolytic activity than those which did not hatch. Such data indicate that after thawing, glycolysis is a major energy-generating pathway for the blastocyst.

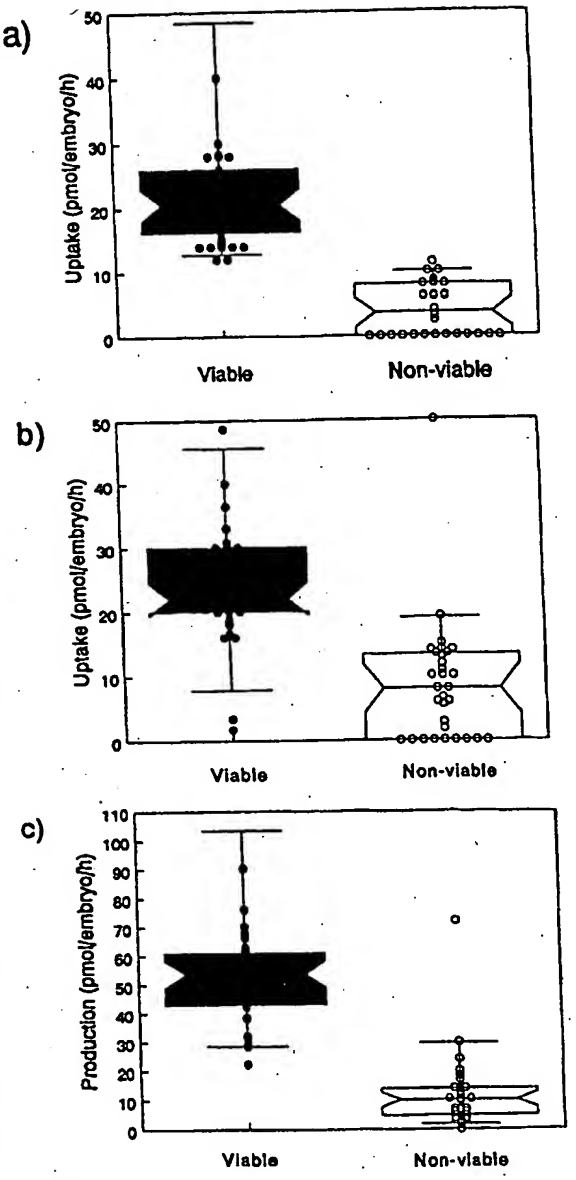

Fig. 2. Notched box plots of glucose uptake (a), pyruvate uptake (b), and lactate production (c) by individual blastocysts after thawing.

This study has demonstrated that energy metabolism of bovine blastocysts is affected by freezing, and that total glucose uptake and its utilization by glycolysis can be used to select viable blastocysts immediately after thawing.

CONCLUSIONS

In conclusion, it is possible to identify, using glucose uptake and lactate production by embryos after thawing,

those blastocysts capable of reexpanding their blastocoels. In contrast, nutrient uptake or utilization could not be used to identify prospectively prior to freezing which embryos would survive thawing. That pyruvate uptake after thawing could not be used to identify those blastocysts which were capable of further development after thawing, together with the observed increase in calculated carbohydrate oxidation, indicates that freezing may induce a partial uncoupling of oxidative phosphorylation. Data indicate that glycolysis is an important energy-generating pathway for the blastocysts after thawing.

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A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability

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Summary. Mouse morulae were exposed to solutions containing 30–50% of permeable agents (ethylene glycol, glycerol, propylene glycol) in modified phosphate-buffered saline (PB1 medium) at 20°C for 20 min. A high percentage of them developed to expanded blastocysts in culture, after exposure to 30% and 40% ethylene glycol (98 and 84%, respectively), or 30% glycerol (88%). Ethylene glycol and glycerol were diluted to 30 and 40% with PB1 medium or with PB1 containing 30% Ficoll or 30% Ficoll + 0·5 M-sucrose, immersed in liquid nitrogen in straws and warmed in 20°C water. Solutions containing 40% of a permeable agent with Ficoll did not crystallize during cooling or warming. Mouse morulae were exposed to 40% ethylene glycol in PB1 medium containing 30% Ficoll (EF) or PB1 medium + 30% Ficoll + 0·5 M-sucrose (EFS) for 5–20 min at 20°C. EFS solution was non-toxic to the embryos during 5 min of exposure. When embryos, equilibrated in EFS solution for 2 or 5 min at 20°C, were vitrified at −196°C and were warmed rapidly, nearly all embryos developed in culture (97–98%), and 51% developed to live young at term after transfer. This method, which results in virtually no decrease in embryonic viability, may be of practical use for embryo preservation.

Keywords: mouse; embryo; vitrification; ethylene glycol; sucrose; Ficoll

Introduction

The embryo freezing technique first reported by Whittingham *et al.* (1972) has been one of the most reliable methods for embryo preservation, and is widely used (Wood *et al.*, 1987). Most attempts to improve the technique, therefore, have been directed to simplification of procedures (Wood & Farrant, 1980; Kasai *et al.*, 1980; Miyamoto & Ishibashi, 1986; Széll & Shelton, 1986a; Trounson *et al.*, 1987). Among such attempts, vitrification (Rall & Fahy, 1985) is a significant advance, in which embyros are suspended in a highly concentrated solution which can be cooled to liquid nitrogen temperaures without crystallizing. However, the initial vitrification solution (VS1) was highly toxic and embryos must be exposed to this solution at ~4°C, which reduces the practicality of the technique. In addition, embryos must be equilibrated in VS1 in a stepwise manner for 20–35 min at 2 different temperatures before being plunged into liquid nitrogen.

Vitrification, however, has potential advantages over conventional methods in that it takes only a few seconds for cooling embryos, and there is no extracellular crystallization, which is one of the major causes of cell injury. Besides simplification of the procedures, the most important requirement of an embryo preservation technique must be to maintain high survival. In this paper, we describe a simple method for embryo storage by vitrification, which results in extremely high survival rates.

Materials and Methods

Embryos. Female ICR mice (6–12 weeks old; CLEA Japan, Inc., Tokyo, Japan) were induced to superovulate by i.p. injections of 5 i.u. PMSG (Serotropin: Teikokuzoki, Tokyo, Japan) and 5 i.u. hCG (Puberogen: Sankyozoki, Tokyo, Japan) given 48 h apart. Females were mated with ICR male mice, except for the embryo transfer experiment, for which C3H males were used. Embryos were flushed from the upper half of the excised uteri and a region of the oviducts with modified phosphate-buffered saline (PB1: Whittingham, 1971) 77–79 h after the injection of hCG. The embryos were washed in fresh PB1 medium and only morphologically normal, compacted morulae were used.

Experiments involving suspension of embryos in cryoprotectant solutions were conducted in a room at 20 ± 0·5°C.

Vitrification solutions. As candidates for a basic agent to compose a new vitrification solution, 3 permeable cryoprotectants, ethylene glycol, glycerol and propylene glycol, were examined. Non-permeable agents were Ficoll 70 (average molecular weight 70 000; Pharmacia, Uppsala, Sweden) as a macromolecule to assist vitrification, and sucrose as a low molecular weight compound which causes embryo shrinkage by osmosis.

Toxicity test of cryoprotectants. In the first series of experiments, the toxicity of individual permeable cryoprotectants was tested. Embryos (9–10) were suspended in 100 µl PB1 medium containing a permeable cryoprotectant at a concentration of 30, 40 or 50% (v/v) under paraffin oil (Nacalai Tesque, Inc., Kyoto, Japan) in a culture dish (35 × 10 mm, Nunc Inc., Naperville, IL, USA) at 20°C. After 20 min, embryos were transferred to PB1 medium containing the same agent (the same concentration) +0·5 M-sucrose, then to PB1 medium containing 0·5 M-sucrose (S-PB1), and then to fresh PB1 medium at 5-min intervals at 20°C.

In the second series of experiments, the toxicity of 2 vitrification solutions was tested. Embryos (9–10) were suspended in 40% ethylene glycol diluted in PB1 medium containing either 30% Ficoll (EF solution) or 30% Ficoll + 0·5 M-sucrose (EFS solution) under paraffin oil in a culture dish at 20°C. Final concentrations of Ficoll and sucrose were, therefore, 18% and 0·3 M, respectively. After holding for 5, 10, 15 or 20 min at 20°C, embryos in EF solution were transferred successively to EFS solution, S-PB1 medium and fresh PB1 medium at 5-min intervals. Those in EFS solution were transferred directly to S-PB1 medium and then to fresh PB1 medium 5 min later.

Recovered embryos were washed in a modified Krebs–Ringer–bicarbonate (KRB) medium (Toyoda & Chang, 1974) and were cultured in ~0·3 ml of the medium under paraffin oil in 5% CO_2 in air at 37°C. The survival of the embryos was assessed by their ability to develop into expanded blastocysts during 48 h of culture.

Experiments were replicated 4–5 times, with 39–50 embryos being tested for each treatment.

Vitrification test of media. Ethylene glycol and glycerol were diluted to a concentration of 30 or 40% with PB1 medium or PB1 medium containing 30% Ficoll or 30% Ficoll + 0·5 M-sucrose. The solutions were introduced into 0·25-ml plastic straws, and were immersed directly into liquid nitrogen. After being held for about 10 sec, they were quickly transferred into 20°C water and agitated gently. According to the appearance of the straws in liquid nitrogen and in 20°C water, samples which remained transparent were considered uncrystallized, and those which turned opaque were considered crystallized.

Experiments were replicated 6 times using two types of plastic straws (Fujihirakogyo, Tokyo, Japan; I.M.V., L'Aigle, France). A solution was scored as crystallizing if the solution turned opaque one or more times.

Vitrification of embryos. EFS solution was prepared in a 0·25-ml plastic straw by successive aspiration of S-PB1 medium (~100 µl), air (~20 µl), EFS solution (~6 µl), air (~6 µl) and EFS solution (~40 µl), with a 1-ml syringe connected by a small silicone tube, and the straw was held horizontally at 20°C (Fig. 1a). Embryos (8–11) were suspended in EFS solution at 20°C in a watch glass, and were washed in the solution once. They were transferred to the larger column of EFS solution (~40 µl), and then air (~6 µl), EFS solution (~6 µl), air (~15 µl) and S-PB1 medium (~20 µl) were aspirated successively (Fig. 1b) before being sealed with the powder. At 2, 5 or 10 min exposure of embryos to the solution, straws were immersed in liquid nitrogen vertically in two steps, in order to prevent bursting of straws: first, about a half of the straw, including the EFS solution, was immersed rapidly, and then the rest of the straw, including the larger column of S-PB1 (~100 µl), was immersed slowly. After storage for 2–125 days, the straws were warmed rapidly in 20°C water. As soon as the crystallized S-PB1 medium in the straw began to melt (about 5 sec), embryos in the vitrification solution were expelled into a watch glass by flushing the straw with ~0·8 ml S-PB1 medium, agitated gently, and then transferred into fresh S-PB1 medium at 20°C. At about 5 min after flushing out, embryos were transferred to fresh PB1 medium.

In-vitro survival of the embryos was assessed as described above. To examine the developmental potential *in vivo*, F1 embryos (C3H × ICR) were vitrified after exposure to EFS solution for 2 or 5 min. They were warmed approximately 12 h in advance of the time when synchrony between embryos and recipient uteri was expected, so that embryos were slightly older than uteri. They were cultured for 1–6 h before being transferred into one or both uterine horns (4–8 per horn (average of 6) and 6–15 per recipient (average of 10·5)) of recipient mice of the ICR strain on Day 3 of pseudopregnancy, induced by mating with a vasectomized ICR male. The recipients were allowed to litter.

Statistics. Data were analysed by χ^2 tests.

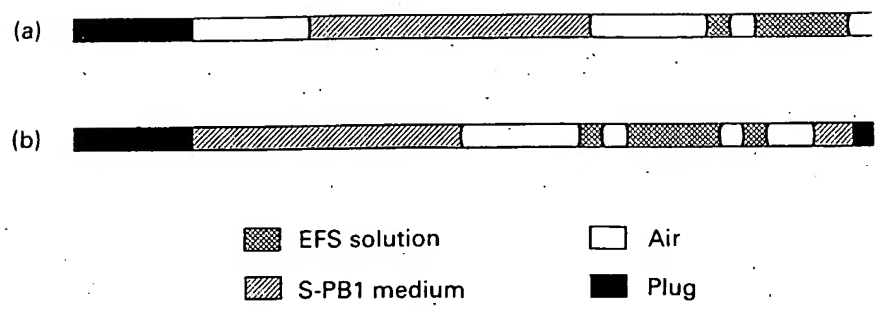

Fig. 1. Diagram of a 0·25-ml straw just before loading embryos (a), and just before cooling in liquid nitrogen (b), showing the configuration of EFS solution, S-PB1 medium, air and plug.

Results

Toxicity test of cryoprotectants

As shown in Table 1, when mouse morulae were exposed to a 30% solution of permeable cryoprotectants for 20 min at 20°C, the percentage of embryos developing to expanded blastocysts in culture was high in ethylene glycol (98%) and glycerol (88%), but was low ($P < 0{\cdot}001$) in propylene glycol (16%). After exposure to 40% solution of permeable agents, only embryos exposed to ethylene glycol showed a high percentage development (84%). However, embryos did not survive exposure to 50% ethylene glycol.

Table 1. Survival of mouse morulae exposed to permeable cryoprotectants for 20 min at 20°C

	No. of embryos developed/no. of embryos treated (%)		
	Concentration of cryoprotectants		
Cryoprotectants	30%	40%	50%
Ethylene glycol	48/49 (98)[a]	42/50 (84)[a]	0/40 (0)[b]
Glycerol	44/50 (88)[a]	1/40 (3)[b]	N.t.
Propylene glycol	8/49 (16)[b]	0/40 (0)[b]	N.t.

Values with different superscripts are significantly different ($P < 0{\cdot}001$).
N.t. = not tested.

Vitrification test of media

As shown in Table 2, when ethylene glycol and glycerol were diluted to 30% with PB1 or PB1 medium containing Ficoll or Ficoll + sucrose, all the media remained transparent after cooling in liquid nitrogen. However, within 1 sec of immersion in 20°C water, straws turned opaque, which is a sign of crystallization. Solutions containing 40% ethylene glycol or glycerol also remained transparent when plunged into liquid nitrogen. At warming, solutions diluted in PB1 medium without non-permeable agents again turned opaque, but media containing Ficoll remained transparent. There was no difference between the effects of ethylene glycol and glycerol as permeable agents on the occurrence of crystallization.

Table 2. Occurrence of crystallization in various media during rapid cooling in liquid nitrogen and warming in 20°C water*

Media		Concentration of permeable cryoprotectants			
		30%		40%	
Permeable cryoprotectants	Diluents	Cooling	Warming	Cooling	Warming
Ethylene glycol	PBI	−	+	−	+
	30% Ficoll in PBI	−	+	−	−
	30% Ficoll + 0·5 M-sucrose in PBI	−	+	−	−
Glycerol	PBI	−	+	−	+
	30% Ficoll in PBI	−	+	−	−
	30% Ficoll + 0·5 M-sucrose in PBI	−	+	−	−

*Transparent samples were considered uncrystallized and were scored (−), and opaque samples were considered crystallized and were scored (+).

Toxicity test of vitrification solutions

The toxicity of vitrification solutions containing ethylene glycol and the impermeable solutes Ficoll and sucrose are shown in Table 3. Survival rates of mouse embryos decreased with increasing durations of exposure in either solution. However, the toxicity of EFS solution was significantly lower than that of EF solution, and virtually no decrease in in-vitro survival of the embryos was observed after exposure to EFS solution for 5 min at 20°C (98% survival).

Table 3. Survival of mouse morulae exposed to vitrification solutions at 20°C

	No. of embryos developed/no. of embryos treated (%)			
	Duration of exposure (min)			
Solution	5	10	15	20
EF	26/50 (52)^{ab}	12/50 (24)^{cd}	0/48 (0)^{ef}	0/39 (0)^{g}
EFS	49/50 (98)^{a}	40/50 (80)^{bc}	39/50 (78)^{de}	10/50 (20)^{fg}

Values with the same superscripts are significantly different (a–a, c–c, d–d, e–e, f–f, $P < 0{\cdot}001$; b–b, g–g, $P < 0{\cdot}01$).

Vitrification of embryos

In total, 274 ICR mouse morulae were vitrified in EFS solution, and 261 (95%) were recovered after warming. When embryos had been equilibrated for 10 min, the proportion of embyros which developed in culture was 77% (Table 4), but when they were vitrified after 2 or 5 min of equilibration, nearly all the recovered embryos were morphologically intact and a high percentage of them developed to expanded blastocysts in culture (97–98%, Table 4). Of 185 vitrified F1 embryos, 178 (96%) were recovered, 175 (98%) of which were morphologically normal and were considered transferable: 167 of them were successfully transferred to 16 recipient mice, 11 of which delivered young (5 embryos were lost during transfer, and 3 were not transferred). Of 112 embryos which had been transferred to recipients that established pregnancy, 57 (51%) developed to live young and 6 (5%) were stillbirths. There was no difference among groups treated for 2 min and 5 min before cooling. All of them had pigmented eyes, confirming that they were derived from vitrified embryos.

Table 4. Survival of mouse morulae stored at −196°C by vitrification in EFS solution

Equilibration period (min)	No. of embryos stored	No. of embryos recovered	No. of embryos developed to expanded blastocysts in culture (%)*
2	104	99	97 (98)[a]
5	111	106	103 (97)[a]
10	59	56	43 (77)[b]

*Percentage of recovered embryos.
Values with different superscripts are significantly different (a–b, $P < 0{\cdot}001$).

Discussion

Vitrification is defined as "the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling" (Fahy *et al.*, 1984). Because the vitrified solution is normally supercooled at a low temperature, it can be crystallized (devitrified) during warming, which leads to the death of the cells suspended in it (Rall, 1987). In order to make a solution vitrify in liquid nitrogen and prevent crystallization during warming, the presence of high concentrations of permeable cryoprotective agents is essential. The first successful solution for vitrifying mouse embryos (VS1; Rall & Fahy, 1985) contained dimethyl sulphoxide, acetamide and propylene glycol as permeable agents. Thereafter, glycerol and propylene glycol were reported effective for vitrifying mouse embryos both in combination (Scheffen *et al.*, 1986) or as single permeating cryoprotectants (Rall, 1987).

For the present experiment on the toxicity of individual cryoprotectants, a selection was made of glycerol and propylene glycol, along with ethylene glycol, which was proved effective for freezing mouse embyros (Miyamoto & Ishibashi, 1977; Kasai *et al.*, 1981). As the toxicity of cryoprotectants is related to temperature, the ambient temperature for embryo manipulation was strictly controlled at 20 ± 0·5°C. The results show that ethyene glycol is the least toxic of the three (Table 1) in spite of the higher molecular concentration of the solution (40% of ethylene glycol, glycerol and propylene glycol are approximately 7·2, 5·5 and 5·5 M, respectively).

Definitive evidence for vitrification requires the use of physical procedures such as X-ray diffraction, calorimetry, or freeze-fracture ultramicroscopy (Rall, 1987), because ice crystals smaller than the wavelength of light will not be detected by visual inspection (Meryman, 1958). However, it has been reported that when several vitrification solutions are cooled and warmed rapidly, there is no evidence of crystallization according to appearance or calorimetry (Rall, 1987). In the present study, therefore, apparent transparency was the criterion for non-crystallization.

It has been shown that the inclusion of a macromolecule in a solution facilitates vitrification (Fahy *et al.*, 1984), and polyethylene glycol is widely used for this purpose (Rall & Fahy, 1985; Rall, 1987). Here, Ficoll 70 was used as a macromolecule, because it has high solubility with low viscosity. The present experiment on vitrification of various media indicates that Ficoll facilitates non-crystallization by preventing devitrification during warming (Table 2). Preliminary data showed that 30% Ficoll in PB1 medium is non-toxic to mouse embryos during 20 min of exposure at 20°C (in-vitro survival was 100%, data not shown).

However, when Ficoll is added to 40% ethylene glycol (EF solution), it increased the toxicity of the ethylene glycol solution: 30% Ficoll (w/v) seems non-toxic but Ficoll occupies about 11% (v/v) of the EF and EFS solutions. If Ficoll were excluded from the EF and EFS solutions, the concentration of ethylene glycol would, therefore, be about 45% in both solutions, which would have increased toxicity. Fortunately, the further addition of 0·3 M-sucrose (EFS solution) decreased this

toxic injury (Table 3). In our toxicity test of the EF and EFS solutions (Table 3), embryos treated in EF solution were suspended in EFS solution for 5 min before removing cryoprotectants in S-PB1, whereas embryos in EFS solution were directly transferred to S-PB1 after treatment. Embryos in the EF solution were therefore exposed to ethylene glycol + Ficoll 5 min longer than those in EFS solution at the same equilibration period. However, survival rates of embryos exposed to EF solution for 5, 10 or 15 min were significantly lower ($P < 0{\cdot}01$, 0·001 and 0·001, respectively) than for those exposed to EFS solution for 10, 15 or 20 min, respectively. Sucrose may reduce toxicity associated with Ficoll by causing the embryos to shrink rapidly and reducing the amount of ethylene glycol in the cells. It has been reported that the addition of sucrose reduces glycerol permeation (Széll & Shelton, 1986b).

After vitrification and warming, in-vitro survival rates of the embryos which had been equilibrated for 5 and 10 min in EFS solution (97 and 77%, respectively, Table 4) were as high as those of embryos exposed to EFS solution for the same periods without cooling (98 and 80%, respectively, Table 3). Therefore, there seems to be no decrease in in-vitro viability by vitrifying embryos. Although the proportion of live young was not very high (51%), it may be possible to elevate it by refinement of the transfer technique, considering the high in-vitro viability.

The new vitrification solution described here (EFS solution) is based on ethylene glycol as the permeating cryoprotectant and two non-permeating agents, Ficoll and sucrose. Although ethylene glycol is less commonly used for embryo freezing than dimethyl sulphoxide and glycerol, the present study indicates that it has an advantage of lower toxicity. Ficoll was found effective for vitrification of embryos for the first time. Sucrose, however, has been successfully used for freezing eggs and embryos in combination with dimethyl sulphoxide (Kasai *et al.*, 1979; Trounson *et al.*, 1987), propylene glycol (Renard *et al.*, 1984), and glycerol (Széll & Shelton, 1986a). Sucrose also facilitates removal of intracellular cryoprotectants during dilution (Leibo & Mazur, 1978; Kasai *et al.*, 1980).

For successful embryo preservation by vitrification, permeation of a cryoprotectant is considered essential, although full permeation is not necessary but rather harmful (Rall, 1987). In the original method of vitrification (Rall & Fahy, 1985; Rall, 1987), embryos are first equilibrated in 25% of a vitrification solution at ~20°C for permeation of cryoprotectants before dehydration at 4°C. In a supplementary experiment, we have observed that mouse morulae suspended in 40% ethylene glycol in PB1 medium at 20°C shrink rapidly and regain most of their volume within 5 min (data not shown), indicating rapid permeation of ethylene glycol into the embryos. In EFS solution, on the other hand, embryos remain shrunken because of the presence of sucrose. As discussed above, sucrose may reduce the amount of intracellular cryoprotectant (Széll & Shelton, 1986b), but in the present conditions, the amount of ethylene glycol which penetrates into the embryos during 2–5 min of equilibration in EFS solution at 20°C seems enough for survival.

Besides giving high survival, this method for embryo preservation, using EFS solution, would be very simple. A time of 2–5 min would be enough to pretreat embryos for equilibration and loading into a straw before plunging into liquid nitrogen. Furthermore, embryos can be manipulated at room temperature (20°C). This new method of vitrification may offer a simple and reliable means for embryo preservation.

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